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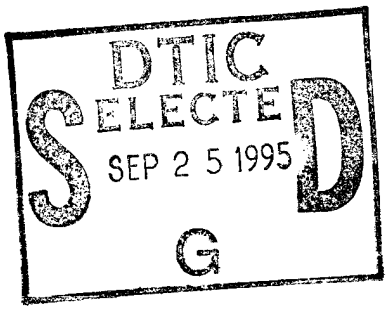

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13. ABSTRACT *(Maximum 200 words)*

Research has been focused on the human cholinesterase genes, their variant protein products and the biological roles of these proteins in different tissue and cell types. To elucidate the biochemical properties and biological functions of this large array of ChE variants, we employed microinjected oocytes and transiently transgenic embryos of *Xenopus laevis*. This has led to the demonstration that the C-terminal peptide characteristic of the brain and muscle form of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) leads to synaptic targeting of these enzymes. Moreover, we were able to show that AChE exerts a morphogenic action on synapse development. The biochemical and biological functions of the BCHE gene and its BuChE protein product were approached by a combination of *Xenopus* oocyte expression and molecular genetics. Substitution of Asp at position 70 in BuChE by Gly creates an enzyme incapable of hydrolyzing succinylcholine, unlike the native enzyme. This structure-function relationship explained the known clinical syndrome of "succinylcholine apnea". The next step was to replace the BuChE peptide harboring Asp70 with that of AChE. This introduced charge modification in the resultant active chimera and explained many of the differences between AChE and BuChE with respect to drug and poison sensitivities. A population diversity study revealed 11% heterozygotes for Asp/Gly70 among Israelis and shed new light on individual variabilities noted for the newly tested anti-AChE drugs, examined for their potential in Alzheimer's therapy. Our current efforts are to extend these studies to elucidate the protective roles of these proteins against organophosphate intoxication, using transgenic mice overexpressing the human ACHE gene.

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Appendix papers published under USAMRDC support

- R. Beeri, A. Gnatt, Y. Lapidot-Lifson, D. Ginzberg, M. Shani, H. Soreq, H. Zakut, "Testicular amplification and impaired transmission of human butyrylcholinesterase cDNA in transgenic mice", Human Reprod. 9 (1994)
- E. Lev-Lehman, A. El-Tamer, A. Yaron, M. Grifman, D. Ginzberg, I. Hanin, H. Soreq, "Cholinotoxic effects on acetylcholinesterase gene expression are associated with brain-specific alterations in G,C-rich transcripts", Brain. Res. 661 (1994) 75-82.
- M. Schwarz, Y. Loewenstein-Lichtenstein, D. Glick, J. Liao, B. Nørgaard-Pedersen, H. Soreq, "Successive organophosphate inhibition and oxime reactivation reveals distinct responses of recombinant human cholinesterase variants", Molec. Brain Res. (in press).
- M. Schwarz, D. Glick, Y. Loewenstein, H. Soreq, "Engineering of human cholinesterases explains and predicts diverse consequences of administration of various drugs and poisons", Pharmacol. Therap. (in press).
- Seidman, R. Ben Aziz-Aloya, R. Timberg, Y. Loewenstein, B. Velan, A. Shafferman, J. Liao, B. Nørgaard-Pedersen, U. Brodbeck, H. Soreq, J. Neurochem. 62 (1994) 167-1681.
- S. Seidman, M. Sternfeld, R. Ben Aziz-Aloya, R. Timberg, D. Kaufer-Nachum, H. Soreq, "Synaptic and epidermal accumulation of human acetylcholinesterase are encoded by alternative 3'-terminal exons", Mol. Cell. Biol. 14 (1995) 459-473.
- H. Soreq, G. Ehrlich, M. Schwarz, Y. Loewenstein, D. Glick, H. Zakut, "Mutation and impaired expression in the ACHE and BCHE genes: neurological implications", Biomed. & Pharmacother. 48 (1994) 253-259.

- R. Beeri, C. Andres, E. Lev-Lehman, R. Timberg, T. Huberman, M. Shani, H. Soreq, "Transgenic expression of human acetylcholinesterase induces progressive cognitive deterioration in mice" (submitted for publication).
- Y. Loewenstein-Lichtenstein, M. Schwarz, D. Glick, B. Nørgaard-Pedersen, H. Zakut, H. Soreq, "Genetic predisposition to adverse consequences of anti-cholinesterase therapies in carriers of the 'atypical' butyrylcholinesterase mutation" (submitted for publication).

Abbreviations

ACh	aceetylcholine
AChE	acetylcholinesterase enzyme
ACHE	acetylcholinesterase gene
AChR	acetylcholine receptor
BCHE	butyrylcholinesterase gene
BuChE	butyrylcholinesterase enzyme
ChAT	choline acetyltransferase
ChE	cholinesterase
CMV	cytomegalovirus
CNS	central nervous system
CSF	cerebrospinal fluid
DFP	diisopropylfluorophosphonate
DIP	diisopropylphosphoryl
DTNB	dithionitrobenzoate
EAIA	enzyme-antigen immumoassay
ELISA	enzyme-linked immunosorbant assay
ICV	intracerebroventricular
NMJ	neuromuscular junction
OP	organophosphate
PAM	pyridine-2-aldoxime methiodide
PCR	polymerase chain reaction
PF	post-fertilization
rHAChE	recombinant human AChE
RT-PCR	reverse transcriptase-PCR
SR	sarcoplasmic reticulum

Amino acid residues are indicated by their single-letter code followed by the sequence position. Amino acid replacements are indicated by the single letter code of the original residue, the sequence position, and the single letter code of the new residue. Thus, the replacement of aspartate at position 70 (D70) by glycine, is indicated by D70G.

Introduction

Acetylcholinesterase (AChE) has long been recognized as an essential component of nerve conduction and muscular innervation. In a grim recognition of this, was the development of anti-cholinesterases (anti-ChEs) as warfare nerve agents and subsequently of pesticides. The search for an antidote was successful in the development of pyridine-2-aldoxime methiodide (PAM), but the search for prophylactic agents has been more problematical. One approach has been to reversibly block AChE before exposure to the nerve agent in order to compete with and thus prevent the irreversible inactivation of the enzyme. Pyridostigmine was used for this purpose during the 1991 Gulf War. Another more ambitious but not yet developed approach has been to produce alternative targets for the nerve agent that might be injected into an individual at risk. The first such alternative to be proposed as protection of the individual's AChE was exogenous human AChE, to be produced by recombinant molecular biological techniques. Later it was found that human butyrylcholinesterase (BuChE) was a more effective alternative target, and that, indeed, it serves normally, as such a decoy target for anti-ChEs of the natural environment.

There is a fine balance of acetylcholine (ACh) production, presynaptic release upon neural stimulation, binding to and activating the acetylcholine receptor (AChR) and termination of action by hydrolysis of ACh. The shift of this balance in disease states (e.g. autoimmunity to the AChR in myasthenia gravis) or due to poisoning (e.g. OP or carbamate pesticide intoxication) clearly has severe consequences. There is also the potential for severe consequences should the balance of ACh production and destruction be altered by an exogenous ChE, and we have been evaluating this possibility by several means, chiefly by introducing a human ChE gene into an animal. Selection of a transgenic animal model is constrained by the availability of technology and by experience. We have used the *Xenopus laevis* tadpole because of our experience with *Xenopus* oocytes, the short time for expression of a gene, cost and the ethical imperative to work with a species as phylogenetically remote as possible from the human. We find that alternative forms of human AChE affect the development of the neuromuscular junction (NMJ) and its ultrastructure differently, and that the nature of the mRNA splicing determines tissue targeting. Because of the limitations inherent in using a non-mammalian model, we have also used the mouse, the mammal in which there is the most experience in introducing human genes. We have used the mouse to observe the effect on body temperature regulation of anti-ChEs, and the protection afforded by the human transgene expressed in the mouse's CNS. We find, also, that in this transgenic mouse, the imbalance of ACh production and destruction results in a progressive loss of cognitive function.

Parallel with these studies on transgenic animals, we have been studying the chemistry of the interaction of anti-ChEs with human AChE and BuChE and also with the major natural variant of human BuChE, especially as this BuChE, the "atypical" variant, appears to offer much less protection against anti-ChEs than normal BuChE. We also evaluated the action of a reactive ACh analog in rat brain, in order to gain some appreciation of the relative vulnerabilities of AChE and BuChE and the dynamics of the brain's recovery from an anti-ChE. That effort also gave us our first experience with differential display PCR, which we continue to exploit.

We continue our efforts to understand the biological roles of BuChE and its functional interaction with AChE, and to exploit this knowledge to develop more effective prophylaxis against warfare nerve agents.

Body of the Report

1. Transgenic *Xenopus* tadpole

Formation of a functional NMJ requires the targeted deposition of synaptic proteins at the nerve-muscle interface (Froehner, 1991; Ohlendieck *et al.*, 1991; Flucher and Daniels, 1989). Among these proteins is AChE. In the developing *Xenopus* embryo, muscle differentiation, primitive neuromuscular contacts, and spontaneous synaptic activity are observed within 1 day post-fertilization (PF) (Kullberg *et al.*, 1977). During the ensuing 24 h, ultrastructural specializations characterizing synaptic differentiation are observed, followed by the acquisition of spontaneous motor activity and hatching (Cohen, 1980). Fervent embryonic development and ultrastructural maturation of the neuromuscular system continue, giving rise to a free swimming tadpole within 4-5 days. From day 2 PF, a steady increase in AChE activity is observed (Gindi and Knowland, 1979), concomitant with a developmentally regulated decrease in the time course of the synaptic potential in *Xenopus* myotomes (Kullberg *et al.*, 1980). We have cloned a DNA sequence encoding human AChE and used it to express catalytically active AChE in microinjected *Xenopus* oocytes (Soreq *et al.*, 1990) and cultured human cells (Velan *et al.*, 1991a). Here, we present a biochemical and histochemical characterization of this recombinant human AChE (rHAChE) expressed in *Xenopus*. We have demonstrated the persistence of overexpressed enzyme in NMJs to at least day 3 of embryonic development and offer evidence indicating subtle alterations in the ultrastructure of NMJs from embryos overexpressing ACHE. Our findings indicate the assignment of catalytically active monomeric AChE to subcellular compartments common to those occupied by native, multimeric *Xenopus* AChE .

Xenopus-expressed AChE is biochemically indistinguishable from native human AChE Microinjected into mature oocytes, 5 ng *in vitro*-transcribed AChEmRNA directed the production of catalytically active AChE displaying substrate and inhibitor interactions characteristic of the human enzyme (Fig. 1A, B). The apparent Km calculated for rAChE towards acetylthiocholine was 0.3 mM, essentially identical to that displayed by AChE expressed in cell lines (Velan *et al.*, 1991b) and native human erythrocyte AChE. In sucrose density centrifugation AChE sedimented primarily as monomers and dimers, although a discernible peak apparently representing globular tetrameric AChE was also observed (Fig. 1C). When plasmid DNA carrying ACHEcDNA downstream of CMVACHE (Velan et al, 1991b) was microinjected into oocytes, active AChE in yields 10-20 fold higher than that observed following RNA injections was obtained (Fig. 1D), demonstrating efficient transcription from this promoter in *Xenopus*.

Transient expression of CMVACHE in *Xenopus* embryos Microinjected into cleaving embryos, CMVACHE directed the biosynthesis of AChE at levels similar to those observed in DNA-injected oocytes, yet, the gross morphology and development of injected embryos appeared normal (Fig. 2). Moreover, gross motor function of microinjected embryos was unimpaired. Microinjected tadpoles survived up to 4 weeks, showing no overt developmental handicaps. Following overnight incubation, at which time embryos had reached the late gastrula stage, endogenous AChE levels were negligible and activity represented a 50- to 100-fold excess over normal (Fig. 3A). From day 2 PF, endogenous AChE activities increased steadily. Using the irreversible AChE inhibitor echothiophate (Neville *et al.*, 1992) to distinguish between endogenous frog AChE and human AChE (Fig. 3A, inset), we observed the persistence of receding levels of human AChE for at least 4 days PF. For the first 3 days, human AChE accounted for >50% of the total measured activity in microinjected embryos and resulted in a state of general overexpression. In immunoblot analysis, human AChE was observed to comigrate with native human brain AChE, yielding a clearly visible doublet band at around 68 kD (Fig. 3B). The doublet may reflect differences in glycosylation (Kronman *et al.*, 1992).

Human AChE remains monomeric in *Xenopus* embryos To examine the possibility that heterologous human AChE undergoes homomeric oligomeric assembly or interacts with either catalytic or non-catalytic subunits of *Xenopus* AChE to produce hybrid oligomers, sucrose density centrifugation and EAIA were performed. At all time points examined, we observed human AChE exclusively as non-assembled monomers sedimenting at approximately 3.2 S, despite the concomitant accumulation of various multimeric forms of the endogenous frog enzyme (Fig. 4). When oligomeric AChE purified from CMVACHE-transfected cell cultures (Velan *et al.*, 1991a) or from human brain (Liao *et al.*, 1992) was preincubated with extracts of day 3 uninjected embryos and similarly analyzed, monomers, dimers, and tetramers were detected, and the distribution of oligomeric forms observed was identical to controls (data not shown).

Subcellular disposition of human AChE in myotomes of CMVACHE-injected embryos Whole mount cytochemical staining of CMVACHE-injected embryos indicated accumulation of AChE in myotomes 2 days PF (not shown). We therefore undertook an ultrastructural analysis. Longitudinal and transverse sections from rostral trunk somites revealed clearly discernible myofibers 2 days PF in both injected and uninjected embryos (Fig. 5). By day 3 PF, both groups displayed significant increases in their numbers of myofibrillar elements and in maturation of the sarcoplasmic reticulum (SR; Fig. 6). To examine the subcellular localization of nascent AChE in transgenic and control embryos we employed cytochemical activity staining (Karnovsky, 1964). Crystalline deposits were observed primarily in association with myofibrils, amidst the myofilaments and within the SR (Figs. 5 and 6). Various organelles, including the nuclear membrane, free and bound polyribosomes, Golgi, and sometimes mitochondria were also observed to be stained (Figs. 5 and 6 and data not shown). At day 2 PF, staining in CMVACHE-injected embryos was more pronounced, both in the quantity and intensity of reaction product (Fig. 5). However, variability was observed between tissue blocks, probably reflecting mosaic expression of the injected DNA and/or variability in the efficiency of expression between embryos. Staining appeared to be concentrated at the I band of myofibers, particularly around the triad marking the intersection of the SR and T-tubule systems. Cross sections revealed especially prominent staining within the SR (Fig. 6A,B). Strong staining was now observed at both the A and I bands, and for the first time, within the T-tubules (Fig. 6C,D). Overall, day 2 CMVACHE-injected myotomes resembled day 3 uninjected control myotomes in staining incidence and intensity (Figs. 5A,C and 6B,D).

Ultrastructural consequences of overexpressed AChE in *Xenopus* NMJs To examine the persistence of overexpression and its implications for synaptic ultrastructure, we studied both cytochemically stained and closely appositioned unstained NMJs from 3 day old injected embryos (Fig. 7 and Table 2). Seventy-two percent of the post-synaptic membrane length (SL/PSL, Table 2) was stained, on average, for active AChE. Moreover, the total area covered by reaction product was approximately 4-fold greater than controls in NMJs from CMVACHE-injected embryos (SA, Table 2). In addition, the staining observed in NMJs from injected embryos was considerably more intense, forming large black accumulations of reaction product (Fig. 7A-B,D-E). Ultrastructural features of NMJs from injected embryos were best discerned in unstained synapses. NMJs from CMVACHE-injected embryos displayed more secondary folds and discrete contacts between pre- and post-synaptic membranes (Fig. 7F). Furthermore, the average post-synaptic membrane length in NMJs from CMVACHE-injected embryos was 30% larger and considerably less variable (SL, Table 2), yet, the distance across the synaptic cleft was both larger and more variable. NMJs overexpressing human AChE thus appeared more developed in their structural buildup.

Despite their lack of MyoD elements, some constructs carrying the CMV promoter were shown to be expressed in myotomes of transgenic mouse embryos (Kothary *et al.*, 1991). Therefore, the characteristic subcellular segregation of overexpressed human AChE in muscle may reflect either tissue-specific biosynthesis or post-translational processing of nascent enzyme present in myotomal

progenitor cells at the onset of myogenesis. The high levels of human AChE present in gastrula stage embryos may argue for the latter possibility. In that case, the cytochemical data indicate the existence of an intrinsic, evolutionarily conserved property directing the subcellular trafficking of AChE in muscle, and thus explain the accumulation of human AChE in NMJs of ACHEDNA-injected embryos. Furthermore, these results may imply that cotranslational processes are not required for the correct compartmentalization of AChE in muscle cells.

The general state of myotomal overexpression induced by microinjection of CMVACHE persisted at least 3 days. The area covered by reaction product in cytochemically stained NMJs from day 3, CMVACHE-injected embryos was 4-5 fold greater than that observed in controls. The apparent reduction in the level of synaptic overexpression from day 2 to day 3 PF may reflect the overall decline in total human AChE activity. However, since this calculation does not consider the higher density staining observed in NMJs from CMVACHE-injected embryos, it represents an underestimate of the actual synaptic AChE content. Therefore, our data indicate enhanced stability of human AChE at the NMJ compared to the total pool, a conclusion consistent with the observation that extracellular matrix-associated AChE persists *in situ* long after denervation of adult frog skeletal muscle (Anglister and McMahan, 1985).

Table I. Subcellular fractionation of rHAChE in CMVACHE-injected *Xenopus* embryos[a]

	rH			
fraction	day 1	day 2	day 3	Fr (day 3)
LSS	57 ± 2	60 ± 4	53 ± 3	36 ±5
DS	37 ± 2	34 ± 4	36 ± 3	31 ± 4
HSS	6 ±2	5 ± 1	10 ± 1	33 ±7

[a]Fertilized *Xenopus* eggs were microinjected with 1 ng of CMVACHEDNA, cultured for 1-3 days, and subjected to homogenization and subcellular frctionation. rHAChE in each fraction (rH) was detected by enzyme-antigen immunoassay (Liao et al., 1992) using a specific mAb raised against bovine brain AChE. Endogenous AChE activity in uninjected tadpoles (Fr) was determined by the standard colorimetric assay. Percent enzyme activity in each fraction (average SEM) is shown for 3 to 5 groups of 3 embryos from a single microinjection experiment. LS, low salt-soluble; DS, detergernt-soluble; HSS, high salt-soluble.

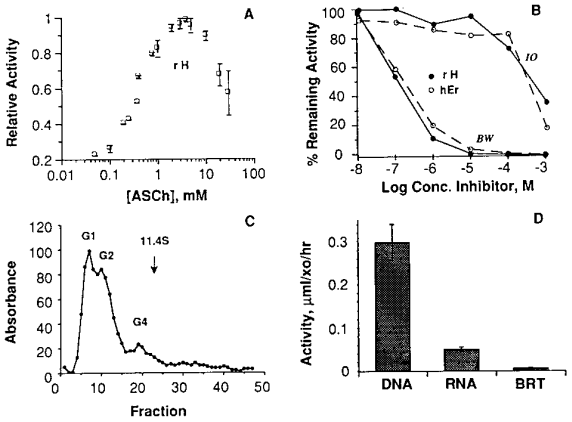


FIG. 1. *Xenopus* oocytes express catalytically active rHAChE. **A:** Inhibition by excess substrate. Mature *Xenopus* oocytes were injected with 5 ng of in vitro-transcribed AChE mRNA (Soreq et al., 1990) and incubated overnight at 17°C. Homogenates corresponding to one-third oocyte were assayed for AChE activity in the presence of various concentrations of acetylthiocholine (ASCh) substrate [average of three experiments ± SEM (bars)]. **B:** Sensitivity to selective inhibitors. Oocyte homogenates were preincubated for 30 min in assay buffer containing the AChE-specific, reversible inhibitor 1,5-bis(4-allyldimethylammoniumphenyl)-pentan-3-one dibromide [BW284C51 (BW)] or the butyrylcholinesterase-specific inhibitor tetraisopropylpyrophosphoramide (IO) at the indicated concentrations and assayed for remaining activity following addition of 2 m*M* ASCh. Data are averages of duplicate assays from two independent microinjection experiments. AChE extracted from human erythrocytes (hEr) served as control. rH, rHAChE. **C:** Oligomeric assembly. Homogenates from ACHE mRNA-injected oocytes were subjected to sucrose density centrifugation as described in Materials and Methods. Data are averages of three experiments. Note that in addition to the free monomer (3.2S; G1), the oocyte appears to generate dimers (5.6S; G2) and to a lesser extent tetramers (10.2S; G4) of human AChE. Endogenous oocyte AChE activity is undetectable under these conditions. The arrow marks the position of bovine liver catalase (11.4S). **D:** Expression of ACHE DNA in *Xenopus*. Oocytes were injected with 5 ng of synthetic ACHE mRNA or CMVACHE (Velan et al., 1991a) and incubated for 1 (RNA) to 3 (DNA) days. Oocytes injected with incubation medium (BRT) or uninjected oocytes served as control. Activity is expressed as micromoles of substrate hydrolyzed per hour per oocyte, in mean ± SEM (bars) values for three independent microinjection experiments.

Table 2. Overexpression of AChE in NMJs of 3-day old CMVACHE-injectied *Xenopus* embryos

Experiment	PSL (µm)	SL (µm)	SL/PL ratio	SA (μm^2)
uninjected	2.57	0.79	0.004	0.156
	3.95	0.79	0.20	0.126
	1.54	0.80	0.06	0.080
	2.35	0.73	0.31	0.082
	1.17	0.44	0.085	0.0-63
	1.60	0.29	0.18	0.056
	1.02	0.29	0.28	0.040
	0.88	0.58	0.65	0.075
average ± SD	1.88 ± 0.93	0.58 ± 0.22	0.22 ± 0.19	0.084 ± 0.038
+ CMVACHE	1.76	1.17	0.66	0.284
	2.50	2.05	0.82	0.331
	2.64	1.91	0.72	0.285
	2.50	2.40	0.96	0.476
	3.50	2.03	0.58	0.396
	1.85	1.66	0.90	0.333
	3.10	1.85	0.60	0.535
	3.23	1.76	0.54	0.289
average ± SD	2.64 ± 0.58	1.85 ± 0.33	0.72 ± 0.15	0.37 ± 0.09
p	<0.01	<0.002	<0.002	<0.002

Eight representative synapses from CMVACHE-injected or control uninjeted embryos were assessed for postsynaptic membrane length (PSL), the sum total length covered by reaction product (SL), the fraction of nerve-muscle contact distance displaying reaction product (SL/PSL), and the total stained area (SA). Average ± SD values are given. Measurements were performed on electron micrographs using a hand-held mapping device.

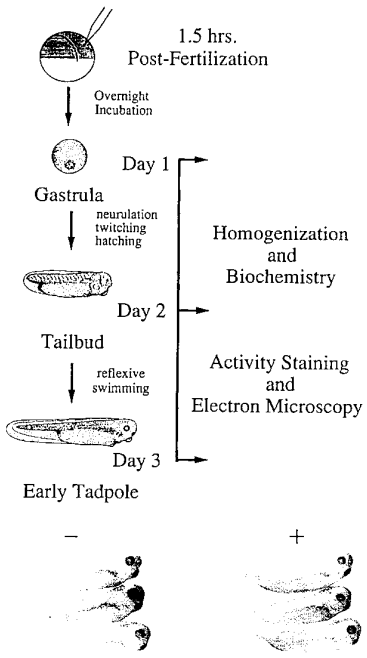


FIG. 2. Normal development of CMVACHE-injected embryos. A schematic representation of a microinjection experiment depicting the principal developmental stages and analytical approaches used in this work is shown together with photographs displaying the normal gross development of unstained microinjected embryos (+) compared with control uninjected embryos (–) 3 days PF.

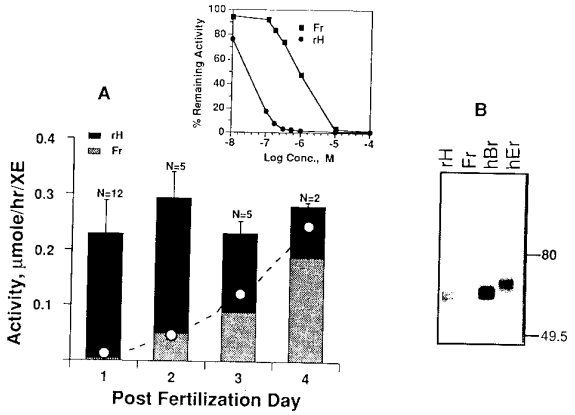


FIG. 3. CMVACHE-injected *Xenopus* embryos express and maintain biochemically distinct heterologous human AChE for at least 4 days. **A:** Overexpression of rHAChE in developing embryos. High-salt/detergent extracts of CMVACHE-injected and uninjected embryos were prepared and assayed for AChE activity in the presence and absence of the selective inhibitor echothiophate (3.3×10^{-7} *M*; **inset**). Endogenous AChE activity was calculated according to an algorithm assuming 90% inhibition of rHAChE and 20% inhibition of frog AChE at this concentration of inhibitor. The bar graph represents the total AChE activity measured per microinjected embryo at various time points following microinjection and the calculated activities attributable to rHAChE (dark shading) and endogenous frog AChE (light shading). The total AChE activity measured in uninjected control embryos at the same time points is indicated by open circles. Data represent average ± SEM (bars) values from four to six embryos from the indicated number (N) of independent microinjection experiments. Inset: Selective inhibition of rHAChE by echothiophate. Homogenates representing endogenous frog (Fr) or recombinant human (rH) AChE were assayed for activity following a 40-min preincubation with the indicated concentrations of echothiophate. Data are averages of three experiments. **B:** Immunochemical discrimination between rHAChE and embryonic *Xenopus* AChE. Affinity-purified AChE from CMVACHE-injected *Xenopus* embryos (rH), control uninjected embryos (Fr), human brain (hBr), and erythrocytes (hEr) was subjected to denaturing gel electrophoresis and protein blot analysis as described in Materials and Methods. Each lane represents ~20 ng of protein, except rH, which contained only 6 ng. Note the complete absence of immunoreactivity with embryonic *Xenopus* AChE, although silver staining of a parallel gel demonstrated detectable protein at the corresponding position (data not shown). The faint upper bands (140–160 kDa) in the lanes displaying native human AChEs represent dimeric forms resulting from incomplete reduction of the intersubunit disulfide bonds (see Liao et al., 1992).

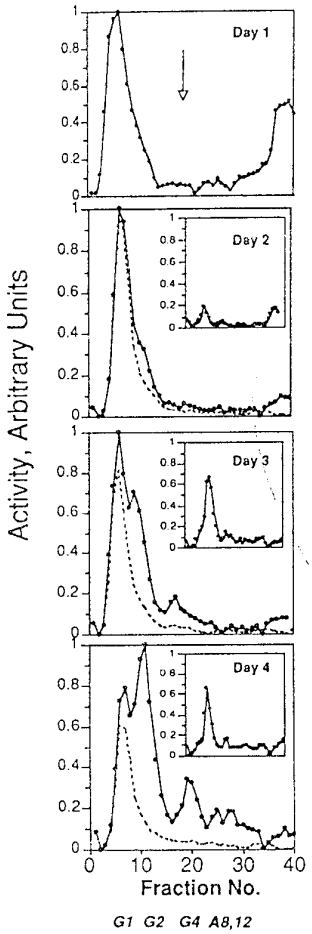


FIG. 4. rHAChE in microinjected *Xenopus* embryos remains monomeric. High-salt/detergent extracts representing two embryos were subjected to sucrose density centrifugation as described in Materials and Methods. Shown are total AChE (solid line) and immunoreactive rHAChE (dotted line) from CMVACHE-injected embryos 1–4 days PF. rHAChE appeared exclusively as a peak representing monomeric AChE (~3.2S) at all time points. The arrow marks the position of bovine liver catalase (11.4S). **Insets:** AChE molecular forms in control uninjected embryos scaled to the total activity levels observed in DNA-injected embryos (see Fig. 2). Peak analysis demonstrated that the distribution of oligomeric forms was identical to that observed in CMVACHE-injected embryos. Note that monomeric AChE is essentially undetectable in control embryos. G1, G2, and G4 indicate the expected positions of the globular monomer, dimer, and tetramer in the gradient; A8 and 12 indicate the positions of "tailed" asymmetric forms. Fraction 0 represents the top of the gradient.

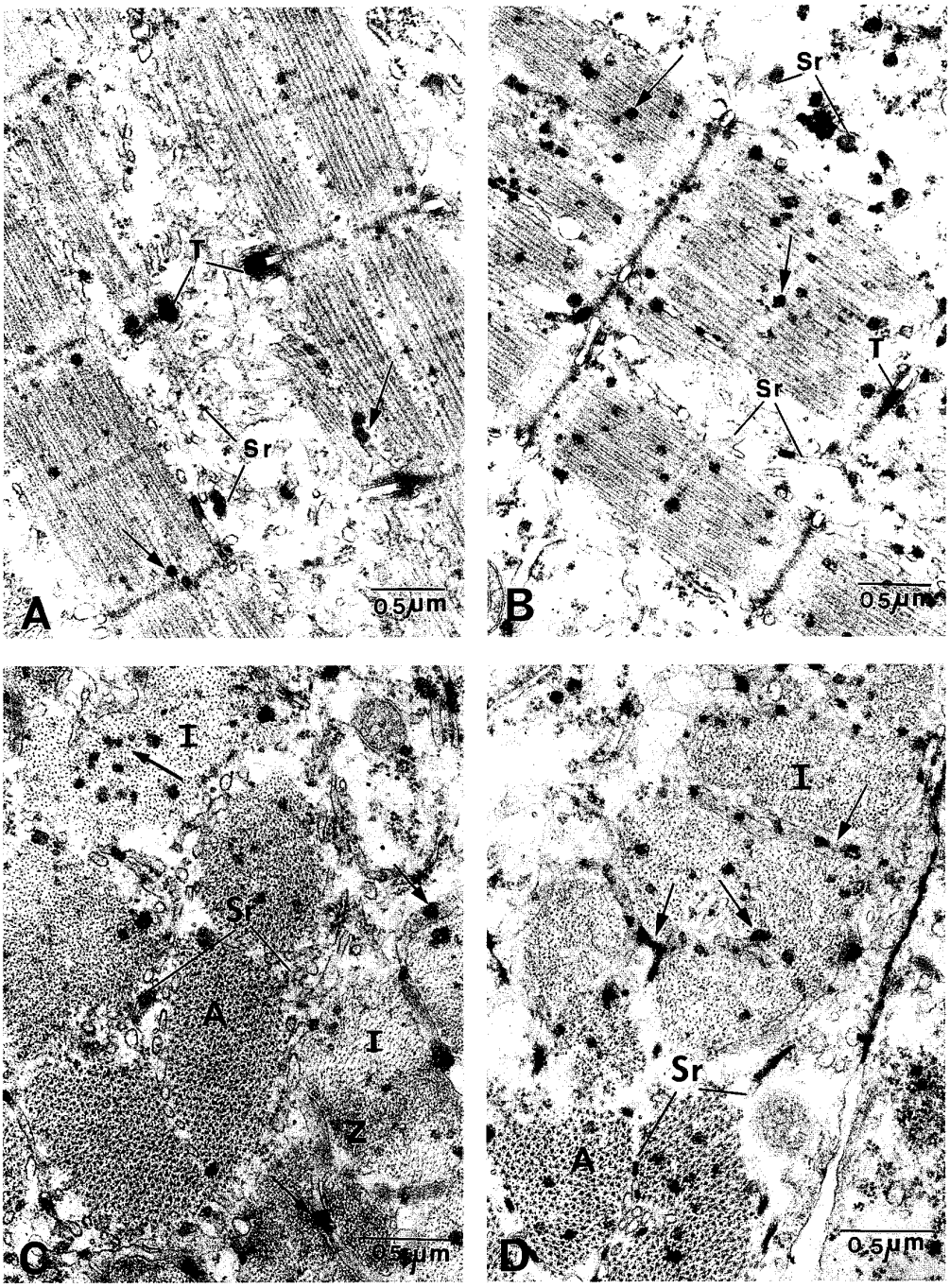


FIG. 6. Overexpression of AChE in myotomes of CMVACHE-injected embryos persists to day 3. Analyses were as in Fig. 5 except that embryos were analyzed after incubation for 3 days. Note the developmental increases in myotomal AChE in both control uninjected (**A** and **C**) and CMVACHE-injected sections (**B** and **D**), especially within the sarcoplasmic reticulum (Sr) and T-tubules (T). Bar = 0.5 μm.

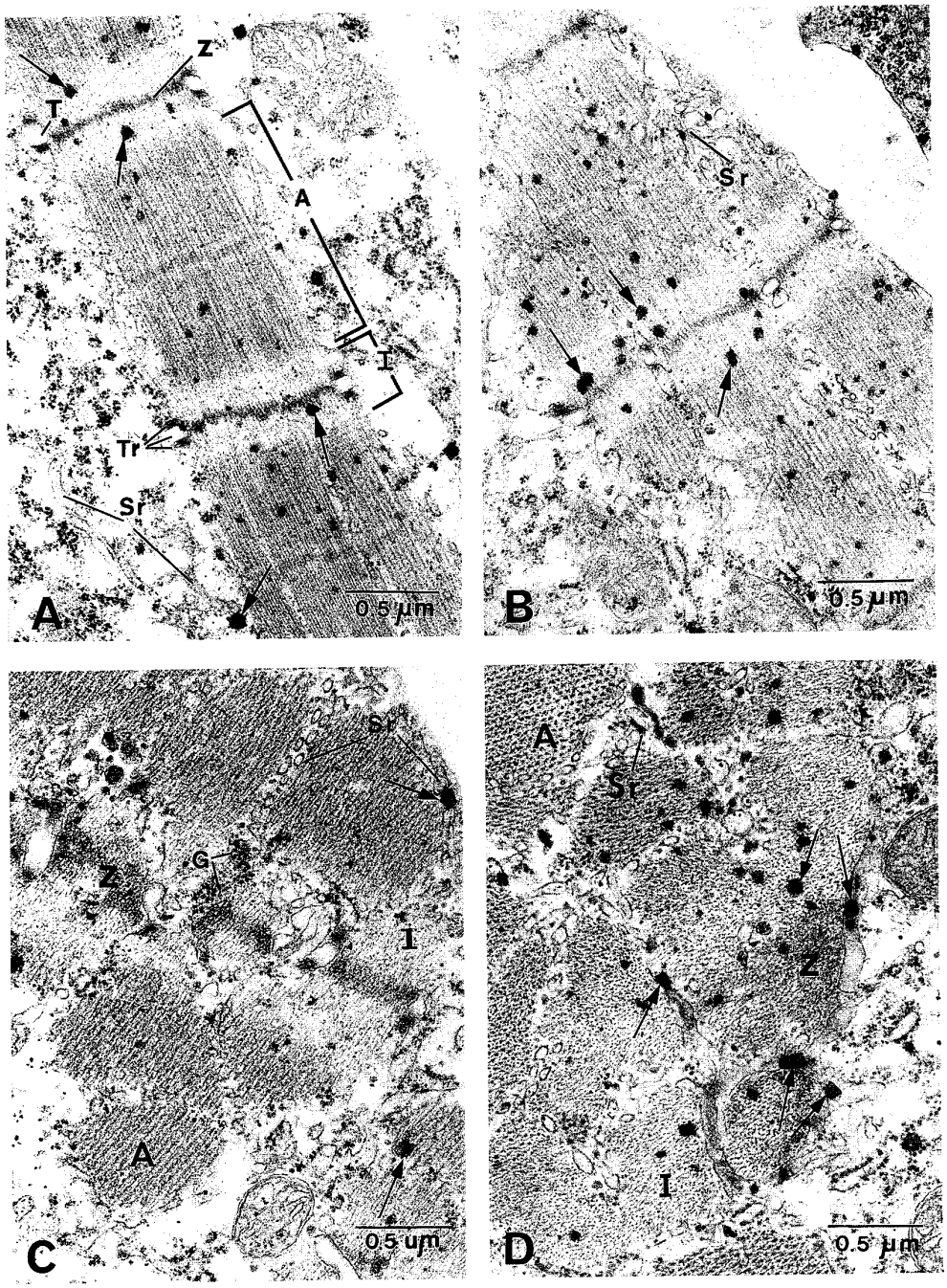


FIG. 5. Disposition of rHAChE in myotomes from 2-day-old microinjected *Xenopus* embryos. Fertilized *Xenopus* eggs were microinjected with 1 ng of CMVACHE, incubated for 2 days at 17°C, fixed, stained, and prepared for electron microscopy as described in Materials and Methods. Uninjected embryos from the same fertilization served as controls and were similarly treated. Arrows mark accumulations of reaction product indicating sites of catalytically active AChE. **A:** Uninjected control myotome in longitudinal section following activity staining for AChE. **B:** Myotome section from CMVACHE-injected embryo. **C:** Uninjected control myotome in transverse section. **D:** Transverse section from CMVACHE-injected embryo. Note the increased intensity of staining in sections from injected embryos versus uninjected controls within the same subcellular compartments, especially within the sarcoplasmic reticulum (Sr). A, A band; I, I band; Z, Z disc; Tr, triad; T, T tubules; G, glycogen particles. Bar = 0.5 μm.

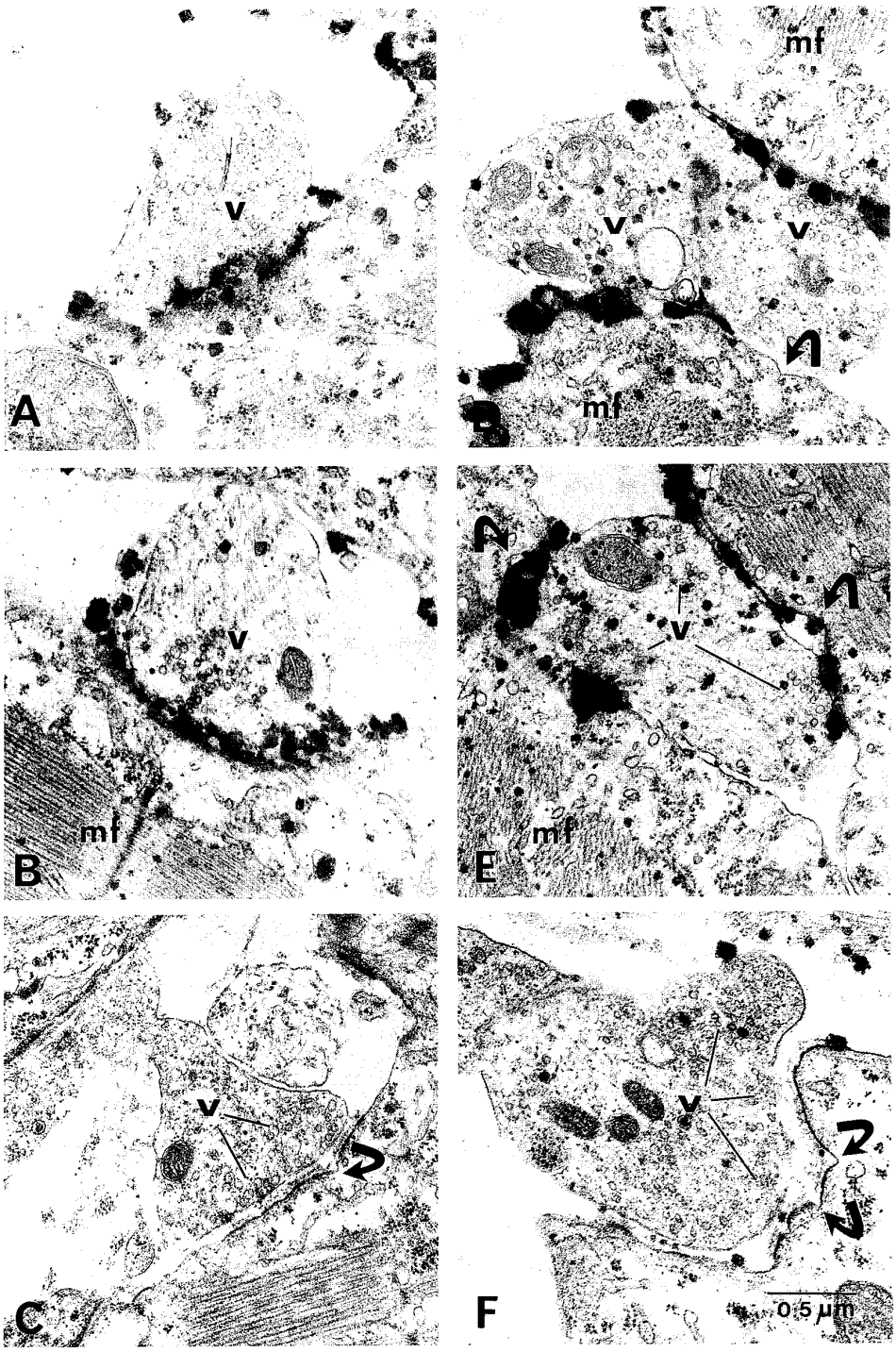


FIG. 7. Structural features in NMJs of 3-day-old CMVACHE-injected *Xenopus* embryos overexpressing AChE. *Xenopus* embryos were cultured for 3 days, fixed, stained for AChE catalytic activity, and examined by transmission electron microscopy as described in Materials and Methods. Two cytochemically stained synapses are presented from uninjected control (**A** and **B**) and CMVACHE-injected (**D** and **E**) embryos. Note the particularly high-density staining in areas directly opposite nerve terminal zones enriched in presynaptic neurotransmitter vesicles (v). **C** and **F:** Representative unstained NMJs from a control and a CMVACHE-injected embryo, respectively. The synapse shown in B represents the highest degree of staining observed in a control section. mf, myofibril. Arrows indicate postsynaptic folds.

2. Transgenic mouse

Low level AChE overexpression is compatible with life The exceedingly low success rates that we found for obtaining viable AChE-transgenic mice suggests that AChE overexpression was compatible with life only at low levels. Seven generations of transgenic mice were raised, all of which presented grossly normal development, activity and behavior.

Transgenic human ACHE expression is limited to CNS neurons Reverse transcription and quantitative PCR amplification (Beeri *et al.*, 1994) revealed both human and mouse ACHEmRNA transcripts in brain regions of the homozygous transgenic mice as is demonstrated in Fig. 1A. Bone marrow and adrenal glands displayed only mouse ACHEmRNAs, with apparently unmodified concentrations of the principal alternative products. The transgene was not expressed in muscle. To associate human ACHEmRNA transcripts with specific brain cell types, we performed *in situ* hybridization experiments. Cholinoceptive hippocampal neurons were intensively labeled, especially in the CA1-CA2 region (Fig. 1B). Thus, expression of the transgene was apparently confined to host CNS neurons that normally express the mouse ACHE gene.

Normally processed transgenic AChE accumulates in cholinergic brain regions AChE from the brain demonstrated unmodified assembly into multimeric enzyme forms (Fig. 1C). Up to 50% of the active enzyme in basal forebrain (Fig. 1C), but none in bone marrow (Table I), interacted with monoclonal antibodies specific to human AChE (Seidman *et al.*, 1995). Catalytic activity measurements in tissue homogenates revealed a 100% increase over control in the detergent-extractable amphiphilic AChE fraction from basal forebrain and more limited increments in cortex, brainstem, cerebellum and spinal cord extracts (Table I). There were no age-dependent changes in this pattern and no differences in the cell type composition of bone marrow of transgenic as compared with control mice. Intensified cytochemical staining of AChE activity was observed in brain sections from transgenic mice in all of the areas that stain for AChE activity in normal mice (Kitt *et al.*, 1994), particularly in the neo-striatum and pallidum (Fig. 2A) as well as in hippocampus (Fig. 2B), the latter two areas associated with learning and memory (Eichenbaum *et al.*, 1990; Yu et al, 1989). Moreover, there were more conspicuous depositions of the electron-dense product of AChE cytochemical staining within synapse-forming dendrites in the anterior hypothalamus from transgenic mice than in control brain sections (Table II). However, synapses interacting with these stained dendrites were of similar length in transgenic mice and in controls (Table II), demonstrating that unlike *Xenopus laevis* NMJs (Shapira *et al.*, 1994; Seidman *et al.*, 1995) AChE overexpression in the mouse brain did not modify the post-synaptic length of at least part of the cholinergic synapses. In addition, there was no sign for neurofibrillary tangles or amyloid plaques in the analyzed brain sections from mice up to 6 months of age.

Transgenic AChE selectively alters hypothermic drug responses In search for the physiological involvement of transgenic AChE, and since cholinergic synapses in the anterior hypothalamus, where we noted AChE overexpression, are involved in thermoregulation (Simpson et al, 1994), we examined hypothermic responses of these transgenic mice to the OP AChE inhibitor paraoxon, the toxic metabolite of the insecticide parathion. Both young and adult transgenic mice were more resistant to paraoxon-induced hypothermia than controls (Fig. 3A, B). The transgenic mice were almost totally resistant to a low paraoxon dose (Fig. 3A). With higher doses, they displayed limited reduction in body temperature and shorter duration of response as compared with controls. Most importantly, transgenic mice exposed to 1 mg/kg dose of paraoxon (Fig. 3B) retained apparently normal physical activity, while control mice experienced under this dose myoclonia and muscle stiffening, symptoms characteristic of cholinergic overstimulation.

Transgenic AChE induces age-dependent decline in spatial learning capacity To examine their cognitive functioning, transgenic and control mice were subjected to several

behavioral tests. When compared to sex-matched groups of non-transgenic control mice at the age of 1, 2-3 and 5-7 months, the AChE-transgenic mice retained normal locomotor and explorative behavior, covering the same space and distance in an open field as their control counterparts. At the same time the open field anxiety of these mice remained unchanged, as evaluated in the frequency of defecation incidents and grooming behavior (Table III). In contrast, conspicuous behavioral differences were observed in these mice in two versions of the Morris water maze (Morris *et al.*, 1981). A different, age-independent defect was observed in the visible platform version of the Morris water maze, in which mice are trained to escape the swimming task by using short-distance cues to climb on a visible platform decorated by a flag and a paper cone (Morris *et al.*, 1981). The transgenics could not improve their performance in this test through training, regardless of their age (Table III). In either case, the early occurrence of this defect at the age of 4 weeks, when the hidden platform test was still correctly performed by these transgenic mice, demonstrates that the defects in the visual and the hidden platform test were unrelated.

The limited neuronal expression and the low copy number of the ACHE transgene ensured that it could cause only subtle cholinergic imbalance. Thanks to the extremely high homology between the human and the mouse enzymes, the transgenic protein was processed and assembled normally. It therefore accumulated in all of the relevant sites, with its highest excess levels found in basal forebrain.

The resistance of these mice bearing the human ACHE gene to the induction of hypothermia by different agents demonstrated modified functioning of cholinergic synapses overexpressing human AChE. Resistance to paraoxon could be expected, as the amount of its target protein, AChE, was significantly increased in the brain of these mice. However, the resistance to muscarinic, nicotinic and serotonergic agonists reflected secondary changes affecting both cholinergic and serotonergic synapses. These changes were caused, either directly or indirectly, by the transgenic enzyme. Yet, no general alterations occurred in the network of neurons controlling thermoregulation, as these transgenic mice retained normal responses to noradrenergic agents and to cold exposure. This suggests loss of specific synapses and/or receptor desensitization as possible causes and indicates the use of cholinergic and serotonergic agonists for early diagnosis of imbalanced cholinergic neurotransmission in human patients.

Transgenic BCHE Gene amplification occurs frequently in tumor tissues yet is, in general, non-heritable. To study the molecular mechanisms conferring this restraint, we created transgenic mice carrying a human BCHE coding sequence, previously found to be amplified in a father and a son. Blot hybridization of tail DNA samples revealed somatic transgene amplifications with variable restriction patterns and intensities, suggesting the occurrence of independent amplification events, in 31% of mice from the FII generation but only 3.5% of the FIII and FIV generations. In contrast, >10-fold amplifications of the BCHE transgene and the endogenous ACHE and c-raf genes appeared in both testis and epididymis DNA for >80% of FIII mice. Drastic, selective reductions in testis BCHEmRNA were detected by the PCR amplification of testis cDNA from transgenic mice, and apparently resulted in the limited transmission of amplified genes.

Table I: Human AChE activities in tissues of control and transgenic mice

	AChE activity[1]			
	Transgenic		Control	
Tissue	Total	Bound to antibodies[2]	Total	Bound to antibodies[2]
Central nervous system				
basal forebrain	402 ± 25	201	187 ± 30	0
cortex	283 ± 18	119	235 ± 12	0
brainstem and cerebellum	95 ± 36	30	88 ± 25	0
spinal cord	255	82	208	0
Other				
adrenal gland	25	0	24.6	0
bone marrow	13.4 ± 0.3	0	17.1 ± 2.3	0

[1]Catalytic activities (nmol acetylthiocholine hydrolyzed/min/mg protein) were determined for detergent-solubilized homogenates. Values are averages of 3 experiments ± standard deviation, except for spinal cord and adrenal gland where single experiments are presented.

[2]Activity after binding to human AChE-specific monoclonal antibodies.

Table II: AChE overexpression does not modify length of axo-dendritic synapses in the anterior hypothalamus of transgenic mice.

Parameters	Controls	Transgenics	p (Student's t test)
Number of synapses[1]	32	12	-
Electron-dense deposits/μm^2[2]	3.4 ± 3.2	6.5 ± 4.0	$p < 0.02$
Post-synaptic length in μm [3]	0.8 ± 0.3	0.7 ± 0.2	ns

[1]Average values ± standard deviations are presented.

[2]Only dendritic electron-dense deposits with rigid limits, reflecting crystal structures, were counted.

[3]Post-synaptic length was measured only for those synapses associated with acetylthiocholine reaction products (i. e. cholinergic synapses). ns = non significant.

Table III: Selective behavioral deficits in AChE-transgenic mice

	Age, months					
	1		2		5-11	
Behavioral parameter	T	C	T	C	T	C
1. Water maze[1]						
a. visual test	33.8 ± 25.0	6.7 ± 1.8	22.5 ± 14.8	5.3 ± 2.2	33.3 ± 12.2	7.3 ± 3.9
b. hidden platform test	17.3 ± 12.5	8.7 ± 3.5	26.4 ± 21.3	14.0 ± 3.5	49.1 ± 27.8	20.5 ± 18.2
2. Explorative behavior[2]						
a. space explored	51.4 ± 19.7	40.3 ± 29.8	62.5 ± 33.6	43.7 ± 21.7	51.5 ± 17.2	54.1 ± 19.4
b. distance walked	11.2 ± 5.1	10.4 ± 8.5	15.9 ± 9.6	11.0 ± 5.4	13.0 ± 5.0	12.2 ± 3.5
3. Open field anxiety[3]						
a. Defecation	1.0 ± 0.6	0.6 ± 0.5	0.1 ± 0.4	0.5 ± 0.8	0.8 ± 0.4	0.4 ± 0.5
b. grooming	1.7 ± 1.0	0.6 ± 1.0	1.7 ± 1.0	1.0 ± 0.9	1.3 ± 1.0	1.2 ± 1.3

[1]Noted are average escape latencies ± standard deviations at day 4 of a Morris water maze test.

[2]Explorative behavior in the open field test was evaluated by measuring the percentage of open field space explored out of 0.36 m^2 and the distance covered in 5 min. Note no significant difference in both parameters between transgenics and controls.

[3] Defecation and grooming events, accepted signs of open field anxiety, were noted.

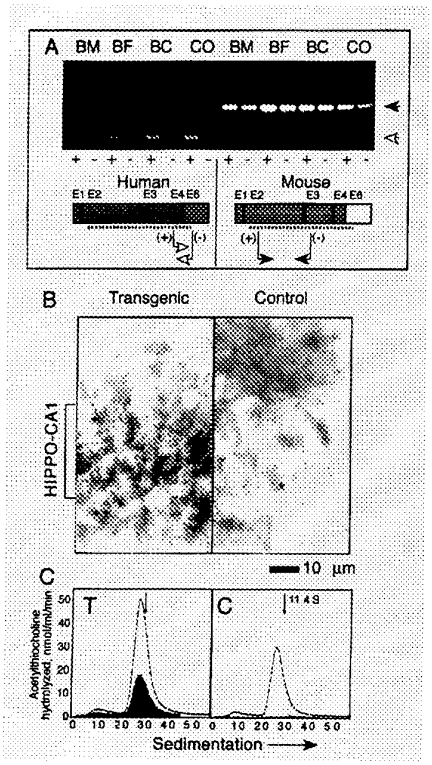


Fig. 1 Detection of transgenic ACHEmRNA and active enzyme.
(A) Identification of human ACHEmRNA. RNA was extracted and amplified from transgenic (+) and control (-) cortex (CO), brainstem and cerebellum (BC), basal forebrain (BF) and bone marrow (BM). Species-specific PCR primers were designed for the indicated positions on the schemes above. Resultant PCR products (275 and 785 bp, respectively) were electrophoresed on agarose gels. Note that expression of HpAChE mRNA was limited to the central nervous system.
(B) *In situ* hybridization. Wholemount *in situ* hybridizations were performed on fixed brain sections from transgenic and control mice. CA1 hippocampal neurons (HIPPO-CA1) in the transgenic, but not the control section were intensely labeled.
(C) Overexpressed AChE activity. Acetylthiocholine hydrolysis levels were determined for tissue homogenates from control (C) and transgenic (T) mice after sucrose gradient centrifugation prior to (clear areas) or following adhesion to human-selective anti-AChE monoclonal antibodies (shaded area). Vertical arrows denote sedimentation of an internal marker, bovine catalase (11.4 S).

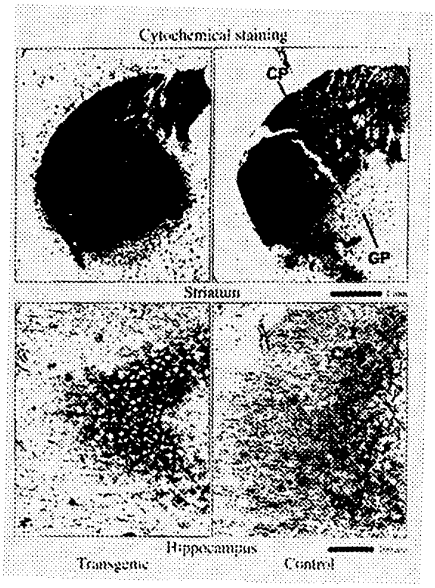


Fig. 2. Neuronal localization of AChE.
A to D: Cells expressing AChE. Wholemount cytochemical staining of AChE activity was performed on fixed sections of control (A, C) and transgenic (B, D) brains. Neuronal cell bodies in caudate-putamen (CP), globus pallidus (GP) (A, B) and hippocampal regions CA2 and CA3 (C, D) were stained more intensely in transgenic than in control brain. Size bars= 1 mm (A,B) or 100 µm (C,D).
E, F: Dendritic accumulation of AChE. Cytochemical staining of AChE activity and electron microscopy were performed on fixed brain sections including axo-dendritic terminals. Excess electron dense reaction products appeared as grains within dendrites (round structures) in transgenic brain (F) as compared with low density labeling in the control brain (E). Size bar= 0.5 µm.

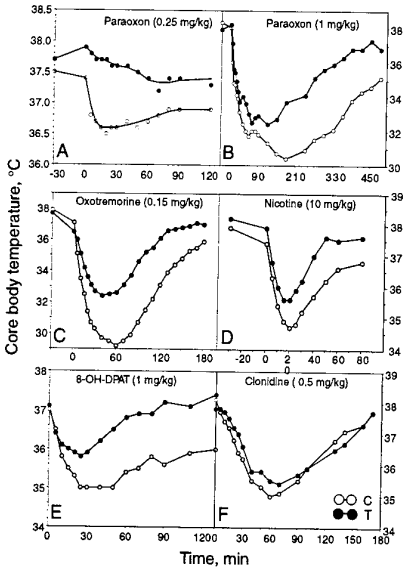


Fig. 3. Transgenic mice display reduced hypothermic responses to cholinergic and serotonergic, but not adrenergic agonists.
Core body temperature was measured in 5 to 7 month old control and transgenic mice at the noted times after intraperitoneal injections of the marked doses of paraoxon (**A, B**), oxotremorine (**C**), nicotine (**D**), 8-OH-DPAT (**E**) or clonidine (**F**). Presented are average data for 5 (**A, F**) or 4 male and female mice (**C, D, E**) or one representative pair out of 3 tested (**B**). Note different time and temperature scales. Temperatures of transgenics at all time points after 10 min from injection were statistically different from those of controls for oxotremorine, nicotine and 8-OH-DPAT (Student's t test, $p < 0.05$). Open circles: control mice, filled circles: transgenic mice.

3. *In vitro* studies of OP and carbamate interactions with AChE and BuChE

Detailed dissection of catalysis by any natural or recombinant ChE variant (Neville *et al.*, 1992; Ordentlich *et al.*, 1993; Soreq and Zakut, 1993; Vellom et al, 1993) should attempt to discriminate among effects on one or another of the several stages of catalysis. As the slowest stage has a half-time on the order of 100 μs (Gilson et al, 1994; Quinn, 1987; Rosenberry, 1975), we have recruited the inactivation of ChEs by an OP (Taylor, 1990) as an analogous reaction in order to study in isolation the first two stages (Fig. 1A). Cleavage of the phosphoryl-ChE bond is extremely slow, making OPs hemi-substrate inhibitors, but the rate of dephosphorylation can be enhanced by PAM (Fig. 1B) (Wilson, 1954), a rigid zwitterionic molecule that juxtaposes its nucleophilic group precisely against the phosphoryl bond, which it displaces. To analyze catalysis on a micro scale and to avoid interference with rate measurements by inactivating or reactivating reagents, we immobilized recombinant *Xenopus* oocyte-produced variant ChEs on selective monoclonal antibodies in multi-well plates and subjected the bound enzymes to successive OP inactivation and oxime-promoted reactivation. By this approach we measured major changes in rates of the reaction with DFP and PAM (Fig. 1B) for a large series of human ChE variants differing within the gorge lining, the acyl-binding site or the C-terminus, always by comparison to the wild-type enzyme forms.

Spatiotemporal dissociation of catalytic steps We collected rate constants for each of the 2 recombinant human enzymes as produced in microinjected *Xenopus* oocytes from the corresponding cloned *in vitro* transcribed mRNA (Neville *et al.*, 1992) or cDNA (Seidman *et al.*, 1994). We also examined a chimera in which the gorge rim, the gorge lining, the conserved oxyanion hole and the choline-binding site of BuChE were substituted with the homologous peptide of AChE (Loewenstein *et al.*, 1993). To test whether variations in the C-terminus (Soreq and Zakut, 1993) would affect catalytic properties of isolated, immobilized ChEs, we used ACHEcDNA vectors, including a vector encoding the major form of human AChE expressed in brain and muscle (Soreq *et al.*, 1990), and designated E6, and an alternative vector, differing in the 3'-terminus and representing the variant ACHEmRNA species expressed in hematopoietic and tumor cells (E5) (Karpel *et al.*, 1994). Several natural and site-directed mutants of recombinant human BuChE completed this series. Each of the recombinant, immobilized enzyme variants was reacted with DFP, followed by regeneration of activity by PAM. The enzymes were enriched by adsorption onto an immobilized antibody (Liao et al, 1993; Seidman *et al.*, 1994). ELISA measurements provided determinations of the amounts of recombinant proteins for evaluation of k_{cat} values.

The gorge lining contributes to enzyme phosphorylation Rates of DFP inactivation were determined for normal BuChE, L286K BuChE and the chimera as compared with the two alternative forms of AChE (Fig. 2A). The second order rate constant for inactivation of AChE by DFP was found to be 160-fold lower than that of BuChE. Introduction of a positive charge into the acyl-binding site of the L286K mutant slowed the inactivation rate of BuChE approximately 8-fold (Fig.2A). In contrast, substitution of the gorge lining by the corresponding AChE peptide, reduced this rate 60-fold (Fig. 2A). In general, inactivation rates of natural BuChE mutants were not severely affected.

Active site charges hamper reactivation The same series of enzymes was further assessed for rates of reactivation . The Kd for DIP-BuChE, 0.3 mM, was close to the value of 0.2 mM found for purified diethylphosphoryl-BuChE (Vellom *et al.*, 1993). The pseudo-first order rate constant for reactivation of DFP-inhibited BuChE (Table I) was 25-fold higher than for either form of AChE (Fig. 2B and Table I). The chimera displayed a reactivation constant only moderately

lower than that of native BuChE. This lower rate for AChE and the chimera was due to a lower true first-order rate constant (k_r), as the affinity for PAM remained unchanged (Table II). In contrast, the BuChE L286K mutant displayed a 40-fold reduction in the pseudo-first order rate constant for reactivation, reflecting a reduction in both reactivation rate and affinity for PAM. This emphasizes the importance of the acyl-binding site in the reactivation process. We further substituted an acidic, a basic, a sulfhydryl and a polar group for F329. Of these, only the sulfhydryl and acidic groups disrupted the reactivation (Table I). For the natural BuChE variants, effects of D70G and Y144H on reactivation were cumulative.

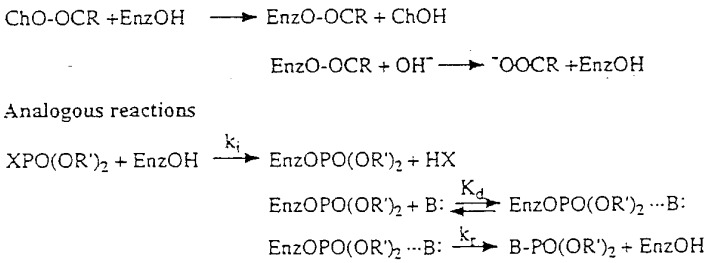
C-terminal variations are innocuous in catalytic terms Both alternative ACHE DNA forms led to enzymes with similar inactivation rates (Fig. 2A and Table I), demonstrating that the natural variability in the C-terminus of AChE contributes to the inactivation rate far less than differences in the acyl-binding site and gorge lining of ChEs.

Effects of variations on kcat The consequences of each variation were further evaluated by determining turnover numbers (k_{cat}, Table I). Effects on kcat in the various mutants were not cumulations of effects on k_i and k'_r, nor are they expected to be, as only the slowest step will be reflected in the catalytic rate. Thus, substitution of L286 with a basic residue reduced reactivation 40-fold but led to only a 5-fold reduction in kcat (Table I). Other replacements at this position, and substitution of the gorge lining in the chimera, had considerably smaller effects on this value.

We chose to focus on three peptide regions of the human ChE molecules: (a) the gorge lining, (b) the acyl-binding site, and (c) the C-terminal peptide in AChE. We found the reactivity of AChE toward DFP to be remarkably lower than that of BuChE, in spite of the close similarity of these two enzymes; our use of the "atypical" and the chimeric enzyme suggested that aromatic gorge-lining residues, especially residues Y72 and Y124, which participate in the peripheral anionic site (Shafferman *et al.*, 1992) contribute significantly toward this difference. Even OP agents that are, unlike DFP, designed as anti-AChE poisons, inactivate BuChE and AChE at similar rates (Raveh *et al.*, 1993), suggesting a special vulnerability of BuChE to OPs. Even in the case of those anti-AChEs, BuChE, with a larger binding site, acts as a scavenger to protect AChE (Raveh *et al.*, 1993). The strikingly lower inactivation rate of the chimera than that of BuChE may be attributable to its having its active site gorge narrowed by numerous aromatic residues. (The chimera's acyl binding site is unchanged.) For the L286K mutation, both inactivation and reactivation are affected. Introduction of a positive charge at the acyl biding site, and possibly also tightly bound water molecules, may disrupt both phases of the reaction. The capacity of AChE to hydrolyze the disfavored substrate, butyrylthiocholine, is reduced to 1/30th the rate of BuChE. The accessibility of BuChE as a serum enzyme raises the possibility that PAM acts by regenerating BuChE and allowing it to react with more of the OP before the OP has a chance to inactivate neuromuscular AChE, in effect turning serum BuChE into an OPase. A finding with important consequences for dealing with OP poisoning is that D70G, by far the most common of the phenotypically different BuChE variants (Whittaker, 1986) reacts with DFP as fast as the normal enzyme, but is reactivated by PAM at only one-fifth the rate. Since D70G also has a 4-fold lower specific activity, than the wild type enzyme (Neville *et al.*, 1990 and unpublished observations), D70G BuChE will have less detoxifying capacity to protect OP-poisoned individuals than its normal counterpart. Moreover, when intravenous PAM administration, in conjunction with other therapies, is used to treat OP intoxication (Taylor, 1990), carriers of D70G BuChE will have a genetic predisposition to poor responses to such therapy. This applies to "atypical" homozygotes, as well as heterozygotes, who together compose up to 7.5% of some populations (Ehrlich *et al.*, 1994). Thus, the recently reported variable efficacy of PAM treatment (Willems *et al.*, 1993) may be due to genetic diversity in the treated patients. Individual differences may further be expected in the sensitivity to poisoning by therapeutic anti-ChEs, such as those used in Alzheimer's disease (Knapp et al;, 1994). Natural

anti-ChEs, such as glyco-alkaloids in *Solanum* plants (Neville *et al.*, 1992) and neurotoxins excreted by cyanobacteria (Carmichael, 1994) may also have diverse effects on these individuals.

A. Catalysis



B.

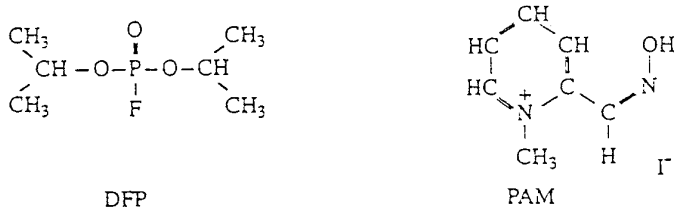


Fig. 1. The experimental paradigm A. Catalysis and analogous reactions In the catalytic cycle the enzyme's active site serine (Enz-OH) displaces the choline moiety of the substrate, forming an acyl-enzyme (covalent) intermediate. The OP agents, being hemi-substrates, act analogously: serine displaces the leaving group (X), forming a dialkyl-phosphoryl-enzyme. Catalysis continues with the hydrolysis of the acyl enzyme, whereas hydrolysis of the phosphoryl-ChE bond is extremely slow. The reactivation rate, however, can be enhanced by nucleophiles (B:) such as choline and PAM, which actively displace the phosphoryl group. B. structure of DFP and PAM

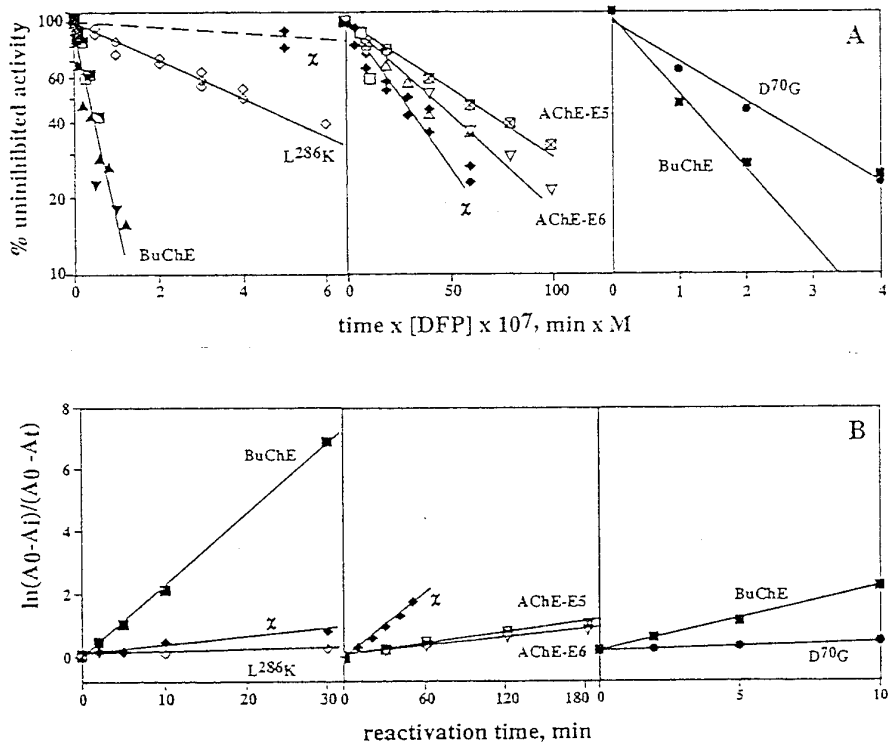


Fig. 2. Inactivation of ChEs and reactivation by PAM A: Data are presented as percent original activity vs. duration of exposure to DFP times the DFP concentration (k_i[DFP]). In the left panel, a comparison of BuChE and its L286K mutant and the BuChE/AChE chimera. In the center panel, a comparison of the natural alternatives of AChE (E6) and AChE (E5), and the chimera, χ. In the right panel, a comparison of representative data for the D70G variant and BuChE. The chimera is presented in the left panel and BuChE in the right panel to assist correlation of the rates of L286K and D70G. BuChE inactivations: ■ 1 nM, ◪ 5 nM, ▲ 10 nM, ▼ 50 nM DFP. AChE (E6) inactivations: □ 0.1 μM, △ 0.5 μM, ▼ 1 μM DFP; AChE (E5) ⊠ 1 μM DFP. L286K BuChE inactivation: ◇ 50 nM DFP. D70G inactivation: ● 5 nM DFP.

B: A logarithmic function of the regain in activity vs. time is presented in the left panel for BuChE ■ the BuChE/AChE chimera ◆, and the L286K mutant ◇ in 1 mM PAM; in the center panel for AChE (E6) ▼, AChE (E5) ⊠, and the chimera ◆ in 0.6 mM; and in the right panel, representative data for BuChE ● and the D70G variant ■ in 1 mM PAM.

4. Failure of "atypical" butyrylcholinesterase to protect against anti-cholinesterases

"Atypical" BCHE, which causes the D70G substitution, is the most common allele of the BCHE gene that causes a clinically variant phenotype. "Atypical" BuChE is incapable of hydrolyzing succinylcholine administered at surgery (Kalow and Davis, 1958; Lockridge, 1990), and is much less sensitive than the normal enzyme to several inhibitors (Neville *et al.*, 1992; McGuire *et al.*, 1989; Kalow and Davis, 1958; Lockridge, 1990). Homozygous carriers of this variant allele -- under 0.04% in Europe but up to 0.6% in certain middle-Eastern populations (Ehrlich, 1994) -- suffer post-anesthesia apnea (Lockridge, 1990) and hypersensitivity to the anti-ChE insecticide parathion (Soreq and Zakut, 1993). Recently, we learned of AB, an individual who had experienced succinylcholine-induced apnea and, during the Gulf War under treatment with pyridostigmine, cholinergic symptoms. Therefore, we initiated a study of the interaction of ChE inhibitors in clinical use or testing (Schwarz *et al.*, 1995a) with serum ChEs from members of this AB's family, as compared to the enzyme from serum and with recombinantly produced variant ChEs (Neville *et al.*, 1992).

BuChE from sera of AB and his father, homozygous and heterozygous carriers of the "atypical" BCHE allele, respectively (Ehrlich *et al.*, 1994), were compared to BuChE from individuals homozygous for the normal allele. To analyze inhibitors that covalently interact with ChEs, we immobilized native human BuChEs through monoclonal antibodies to multiwell microtiter plates (Schwarz *et al.*, 1995b). Recombinant *Xenopus* oocyte-produced variant ChEs, including normal and "atypical" BuChEs and brain- and blood cell-characteristic AChEs (Seidman *et al.*, 1995) were also subjected to inactivation by anti-ChEs and to subsequent spontaneous reactivation (Schwarz *et al.*, 1995b).

Activity of "atypical" BuChE The activity of "atypical" BuChE was found to be about 3-fold lower than normal in AB's serum, which contained a normal amount of enzyme protein. Heterozygotes presented intermediate specific activities. "Atypical" BuChE reacts much slower than does normal with four carbamates, pyridostigmine (Keeler *et al.*, 1991), physostigmine (Giacobini, 1991), heptyl physostigmine (Iversen, 1993) and SDZ-ENA 713 (Enz *et al.*, 1993) (Table IA).

Rate of inhibition of normal and "atypical" BuChE Inhibition by the reversible Alzheimer's disease drug tacrine (Winker *et al.*, 1994) was examined (Table IB). To mimic the heterozygous state, we tested 1:1 mixtures of oocyte homogenates, expressing recombinant normal and "atypical" enzyme (Fig. 1B). In both cases, we observed a drastic reduction in the capacity of "atypical" BuChE to interact with tacrine. Analysis of the relevant site in the enzyme's three-dimensional structure (Harel *et al.*, 1993), further revealed that the distance from the Asp70 carboxyl group to the tacrine anilinic nitrogen in BuChE is only 5.2 Å indicating the possibility of a salt bridge. This interaction is removed in "atypical" BuChE, reflected in a two orders of magnitude increase in tacrine's IC_{50}.

Rate of reactivation of normal and "atypical" BuChE The reactivation rates of AChE and BuChE differed for several drugs, with the decreasing order SDZ-ENA 713, heptyl physostigmine, pyridostigmine and physostigmine (Table IC). However, for no drug was there a dramatic difference between the blood and brain forms of AChE or between normal and "atypical" BuChE (Table IC), which indicates that the differential drug responses of "atypical" BuChE occur at the initial scavenging step.

The possibility that anti-ChE therapies may cause adverse reactions in individuals with variant BCHE genotypes, becomes a pertinent issue in view of the administration of pyridostigmine

bromide to over 400,000 Gulf War soldiers. In homozygotes and possibly in heterozygous carriers of the "atypical" BCHE allele, the lower capacity of blood BuChE to interact with and detoxify some of the drug should result in larger effective doses. "Atypical" homozygotes with practically none of the normal protective detoxifier, would hence become most vulnerable to AChE inhibition under treatment by any anti-ChE drug. Several Alzheimer's disease drugs emerged in this study as much faster inactivators of BuChE than of AChE, suggesting that when administered to patients, these drugs will interact primarily with plasma BuChE.

Table I. Deficient interaction of "atypical" butyrylcholinesterase with various inhibitors

	BuChE		AChE	
	normal	"atypical"	brain-type (E6)	blood cell-type (E5)
A: second order inactivation rate constants ($M^{-1}min^{-1}$)[a]				
pyridostigmine (10^{-5} M)	1.9 ± 0.2	<0.8	22 ± 9	25 ± 7
physostigmine (10^{-6} M)	380 ± 160	26 ± 9	ND	ND
heptyl physostigmine (10^{-8} M)	11000 ± 3,000	770 ± 440	1600 ± 700	1400 ± 400
SDZ-ENA 713 (10^{-5} M)	14 ± 3	0.47 ± 0.46	4.3 ± 1.8	3.3 ± 0.6
B: IC_{50} values (μM)[b]				
tacrine (recombinant)	0.054 ± 0.036	11.4 ± 1.4	0.15 ± 0.08	0.15 ± 0.04
tacrine (serum)	0.082 ± 0.009	8.9 ± 2.5	------	------
C: Time dependent reactivation[c]				
pyridostigmine	9	8	32	24
	17	12	67	54
heptyl physostigmine	13	13	3	3
SDZ-ENA 713	2	1	8	4

[a]Pseudo first-order rate constants were extracted from data such as those in Fig. 1, but for the recombinant enzymes, and with the reagent concentrations indicated in parentheses, and were fit to a first order decay model using the least squares approach. Averages and standard deviations of at least 4 determinations are presented. ND, not determined because of the exceedingly fast reactivation rates.

[b]IC_{50} values of tacrine were measured for recombinant human ChEs and for sera in the presence of 1 mM butyrylthiocholine for BuChE or 1 mM acetylthiocholine for AChE. Values are calculated by GraFit 3.0 (Erithacus Software, Staines, UK). ND, not determined. The data shown are averages and standard deviations of 2 serum samples or 3 recombinant enzyme samples.

[c]Spontaneous reactivation of recombinant human ChEs was examined after complete inhibition of the immobilized enzymes, followed by removal of unreacted inhibitor. Values of percent regained activity after 30 min are shown (average of two experiments).

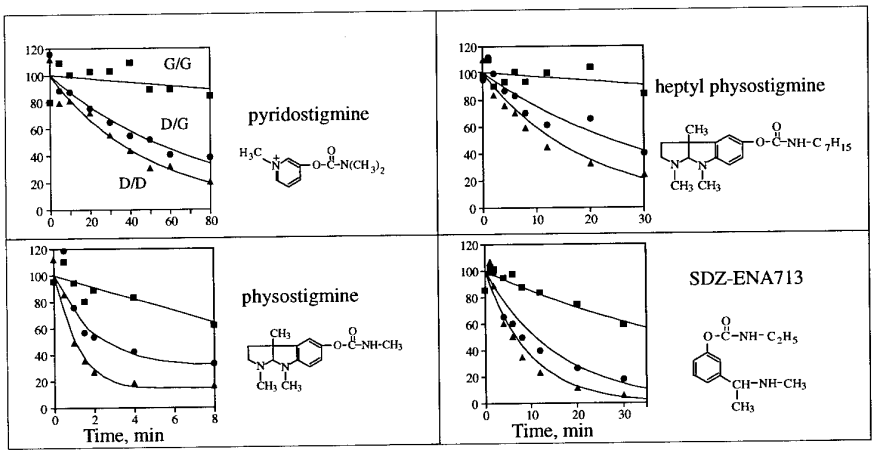


Fig. 1. Inhibition of BuChE variants by anti-ChEs.
A. Differential inactivation kinetics of variant Human sera BuChEs with carbamate inhibitors. Percent original activity as a function of time of exposure to 4 carbamates is presented for a representative experiment. Activities of immobilized ChEs were determined in 10 mM BTCh following incubation for the noted times with the covalently binding inhibitors pyridostigmine (10 μM), physostigmine (1 μM), heptyl physostigmine (0.01 μM), and SDZ-ENA 713 (10 μM). The lines are best fits of the data to first order conditions. ▲, normal, Asp70, BuChE homozygote (D/D); ■ "atypical", Gly70, homozygote (G/G); ●, BuChE heterozygote (G/D).
B. Inhibition of serum and recombinant BuChE by tacrine. In the left panel, data for 3 representative serum types inhibited by tacrine: serum of the propositus, A.B., a homozygote for the "atypical", Gly70, BuChE variant (G/G); of his father, a heterozygote for this variant (G/D); and of a normal homozygote with Asp70 (D/D). In the right panel, tacrine inhibition is observed on equivalent total amounts of recombinant normal (D/D) and "atypical" (G/G) BuChE, and a 1:1 mixture of the two (D/G).

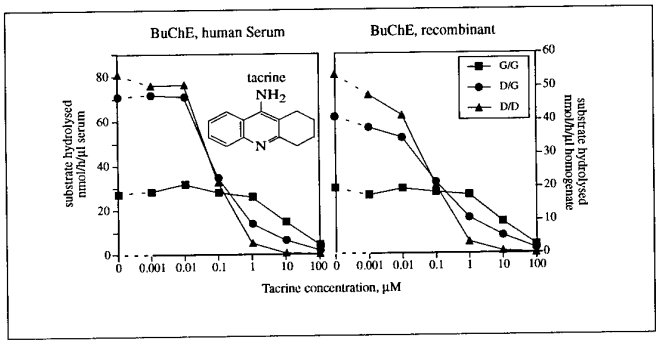


Fig. 2. Inhibition of serum and recombinant BuChE by tacrine. In the left panel, data for 3 representative serum types inhibited by tacrine: serum of the propositus, A.B., a homozygote for the "atypical", Gly70, BuChE variant (G/G); of his father, a heterozygote for this variant (G/D); and of a normal homozygote with Asp70 (D/D). In the right panel, tacrine inhibition is observed on equivalent total amounts of recombinant normal (D/D) and "atypical" (G/G) BuChE, and a 1:1 mixture of the two (D/G).

5. Cholinotoxic effects on rodent brain gene expression

Cholinergic deficits have been associated with several neurodegenerative disorders such as Alzheimer and Huntington's diseases (Wurtman, 1992), suggesting that finely balanced cholinergic metabolism contributes to the maintenance of CNS circuits. An animal model in which a selective cholinergic deficit has been induced is the ethylcholine aziridinium (AF64A) treated rat (Fisher *et al.*, 1982). AF64A is structurally similar to choline, with the distinction that it possesses an ethyl moiety and the highly reactive aziridinium ion. It is taken up by cholinergic neurons via the high affinity choline transport system (Manitone *et al.*, 1981) and causes a specific and long-lasting reduction in the concentration and the activity of cholinergic pre-synaptic biochemical markers (Manitone *et al.*, 1981; Sandberg *et al.*, 1984), including ACh, AChE, ChAT (Leventer *et al.*, 1987), and the high affinity transport system for choline, which is the rate limiting step in ACh synthesis (Murrin, 1980). In addition, rats treated with AF64A also show behavioral cognitive deficits, suggesting that loss of synapses has occurred (Chrobak *et al.*, 1988). Like other aziridinium compounds, AF64A was also found to react also with DNA, to induce DNA damage and to cause premature termination of RNA transcription *in vitro*. Thus, the cholinotoxic effects of AF64A could be attributed to its inhibitory action on cholinergic enzymes activities, to interference with the synthesis of such enzymes, to its DNA damaging activity on G,C-rich genes or to all of these actions together.

AChE and the closely related enzyme BuChE are highly similar in their amino acid sequence and in their computer-modeled three dimensional structure, yet vary in their substrate specificity and in their interaction with inhibitors. In addition, the rat ACHE gene is G,C-rich (Legay *et al.*, 1993) like its human homolog (Soreq *et al.*, 1990), with high predicted sensitivity to guanine-binding agents, whereas the BCHE gene is A,T-rich (Prody *et al.*, 1987) and may be expected to be less sensitive to such agents. This difference made the two ChE genes appropriate models for *in vitro* studies on the effectiveness of AF64A on cholinergic gene expression and/or enzymatic activities. In parallel, we further examined the effects of ICV administration of AF64A on cholinergic enzyme activities and determined the *in vivo* effect of AF64A on ChEmRNA levels by reverse transcription coupled with DNA amplification (RT-PCR) in different rat brain areas. To compare the pattern of expression of G,C-rich genes in brains from AF64A treated and control rats, differential PCR-displays (Liang and Pardee, 1992) were prepared with an arbitrary G,C-rich primer (Welsh *et al.*, 1992). Our findings suggested a consistent correlation between the cholinotoxic effects of AF64A on G,C-rich sequences *in vitro* and *in vivo* and the use of this approach for identifying the target genes to cholinotoxic agents.

AF64A modulates cholinergic enzyme activities *in vivo* AF64A effects on different cholinergic markers *in vivo* were measured in a time-dependence study. To address the cholinergic deficits following ICV administration of 2 nmol AF64A, we determined the enzymatic activities of AChE and ChAT in different brain regions. Septal AChE showed a slight but significant reduction which occurred late, while striatal AChE activity remained apparently unchanged until day 60. In contrast, AChE activity in hippocampus was reduced at day 7 through 60 post-AF64A administration (Fig. 1). The enzymatic activity of ChAT showed a different pattern: septal ChAT activity was significantly increased on day 7, returned to normal level by day 14 and decreased by day 60 post-AF64A treatment. In the striatum, ChAT activities remained normal after 0.5, 1.0 and 2 nmol/side AF64A at all of these time points (Fig. 1, and El-Tamer *et al.*, unpublished data). However, hippocampal ChAT activity was significantly decreased at day 7 through 60. The biochemical measurements thus revealed a particular long-term vulnerability to AF64A effects for cholinergic enzyme activities in the hippocampus.

Differential *in vitro* inhibition of ChEs by AF64A We analyzed enzyme activity following preincubation of AF64A. Our measurements fully reflected the interaction of the released thiocholine product with DTNB, and the color reduction under these experimental conditions was due to enzyme inhibition alone (Hanin *et al.*, 1994). Following incubation of AChE and BuChE with AF64A a reduction of the activity of these enzymes was observed (Fig. 2, top). At the high concentration of AF64A used *in vitro*, one would expect susceptibility to nucleophilic attack by ChE activities. Moreover, at the physiologically-effective average AF64A concentration of 5-20 mM, neither of the enzymes should be inhibited. Therefore, we next focused our investigation on measurements at the mRNA level.

Pretreatment of cholinesterase cDNAs with AF64A causes differential damage to *in vitro* transcription To compare the sensitivity of the ACHE and BCHE genes to AF64A, plasmid DNAs carrying each of these ChE coding sequences were subjected to *in vitro* transcription following pre-incubation with AF64A. Reductions were observed in the yields of RNA transcripts from both ACHE and BCHEcDNA, however to different extents. The ACHE gene therefore displayed differential sensitivity over that of the BCHE gene toward AF64A toxicity *in vitro*, which predicted that physiologically effective concentrations of this cholinotoxin may modulate transcriptional activities of the corresponding genes in cholinergic and/or cholinoceptive cells.

AF64A administration modulates ChE mRNA levels *in vivo* To assess the *in vivo* levels of CHEmRNAs, total RNA extracts from septum, hippocampus and striatum from AF64A-treated rats were subjected to reverse transcription followed by kinetic follow-up of PCR amplification using primers specific to each of the ChE genes (RT-PCR). This analysis revealed that the amounts of septal and striatal ACHEmRNA were reduced, while BCHEmRNA levels remained unchanged (not shown). In contrast, hippocampal ACHEmRNA was higher in treated rats at day 7 post-injection and returned to apparently normal levels at day 60. In this same region, BCHEmRNA was reduced by 70% on day 7 and remained as low as 50% of control on day 60 (Fig. 3). Although AChE activity was significantly reduced in the hippocampus, the RT-PCR analysis revealed a concomitant and pronounced increase in ACHEmRNA, unique to this brain region.

AF64A induces region-specific alterations in the differential PCR display of G,C-rich transcripts To examine whether the increased levels of ACHEmRNA in hippocampus reflected a general change in transcription of G,C-rich genes, we employed the approach of differential PCR display. The general pattern of displayed products did not change in the AF64A-treated rats as compared to control rats (Fig. 4). While no AF64A-dependent changes could be observed in striatal mRNA, we were able to detect several quantitative changes in PCR products displayed from hippocampus and septum following AF64A administration. In the septum the levels of 3 transcripts were decreased on day 7 and remained low at day 60 (Fig. 4). At least 3 other PCR fragments appeared to be stronger in the treated hippocampus 60 days post AF64A. This implies that the increase of ACHEmRNA in the hippocampus was consistent with an increase in multiple G,C-rich transcripts which was particular to this cholinoceptive brain region.

The *in vivo* experiments were complemented by a series of *in vitro* tests. *In vitro* inhibition of transcription of G,C-rich ACHEcDNA was achieved at concentrations of AF64A that were 2 times lower than those required to inhibit the A,T-rich BCHEcDNA, attributing at least part of the *in vivo* changes in ACHE gene expression to its G,C-rich composition. *In vitro* studies further revealed that AChE activity was more sensitive to this alkylating agent than that of BuChE, and that this inhibition required higher AF64A concentrations than those generally administered ICV. Moreover, access of such compounds to intracellular enzymes in the CNS parenchyma in the *in vivo* situation is very limited. Therefore, our *in vitro* tests suggest that to achieve direct inhibition of enzyme activities, the intracellular concentration of AF64A should be higher than those present in the CSF.

The *in vivo* inhibition of AChE activity thus suggested that ICV-administered AF64A actively accumulated in cholinergic synapses and entered into cholinergic and/or cholinoceptive cell bodies, where it could reach higher local concentrations. Our findings therefore support the theory of active uptake into cholinergic cell bodies via the choline transport system, which was previously suggested as a mechanism of action for AF64A (Manitone *et al.*, 1981; Sandberg *et al.*, 1985). Once in these cell bodies, AF64A is likely to interact with G,C-rich genes such as ACHE and interfere with their transcription (Futscher *et al.*, 1992). Moreover, the theory of active uptake further explains the apparent direct reduction of protein activities. Altogether, these findings imply that penetration of this alkylating agent into the brain can induce a to multileveled cholinergic damage. This includes mechanisms of nucleophilic attack and blocking of guanines in the DNA at cholinergic cell bodies on the one hand, and interference with specific protein subsets at cell bodies and nerve terminals on the other hand.

At the level of mRNA, the main damage induced *in vivo* by AF64A appeared to be region-specific and partially transient. At the protein level we could discriminate between the vulnerable hippocampus and the more resistant septum and striatum. Injection of 2 nmol AF64A/ventricle did not affect the activities of cholinergic markers measured in the striatum, which was previously found to be sensitive to higher doses of this cholinotoxin (Sandberg *et al.*, 1984). In contrast, the mRNA levels of both ACHE, b-actin and other unidentified G,C-rich genes were significantly increased in the hippocampus by 7 days post AF64A administration. This indicated that the *in vivo* decrease in AChE activities in the hippocampus was secondary to the reduction in ACHEmRNA in the septum, projecting to the hippocampus, and that this change caused a feedback increase of transcription of several genes, including ACHE and b-actin, in this cholinoceptive and vulnerable area.

To evaluate the general transcriptional damage caused by AF64A, we employed differential PCR display. Differentially expressed mRNAs were indeed observed in septum, striatum and hippocampus, and some of those particular to the hippocampus were modulated following AF64A administration. The long term changes in gene expression detected by the PCR display suggest the use of this approach to clone and identify the modulated transcripts. That both the protein activities and the PCR display changes were long term can further indicate damage to the machinery of mRNA translation. This would reduce AChE activities even under conditions where ACHEmRNA levels returned to normal, as was the case by day 60 post AF64A treatment. The transcriptional changes in AF64A-treated hippocampus are in line with the behavioral effects induced by this cholinotoxin.

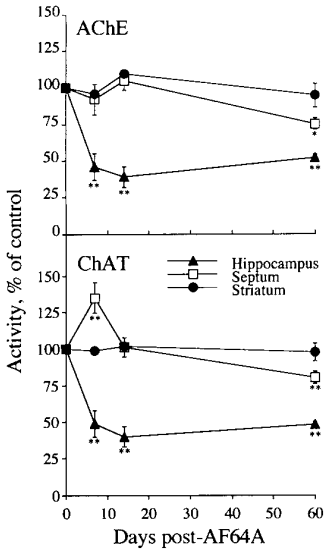


Fig. 1. In vivo effects of AF64A on cholinergic markers. Time-dependent effects of AF64A (2.0 nmol/side) on AChE and ChAT activities are presented for septum, hippocampus and striatum. Animals were sacrificed by decapitation at the indicated time after i.c.v. injection of AF64A. Enzymatic assays were performed in duplicate on tissue homogenates, all as detailed under Experimental Procedures. Data represent the average (mean ± S.E.M., $n = 3$ rats per group) of enzymatic activity expressed as percent of control. AChE activity in control group: mean ± S.E.M., 3476 ± 228, 9214 ± 281 and 20876 ± 474 nmol/mg/h in hippocampus, septum and striatum, respectively. ChAT activity in control group: mean ± S.E.M., 42.1 ± 3.5, 66.5 ± 5.2 and 209.7 ± 7.54 nmol/mg/h in hippocampus, septum and striatum, respectively. * $P < 0.05$; ** $P < 0.01$, compared to vehicle treated group.

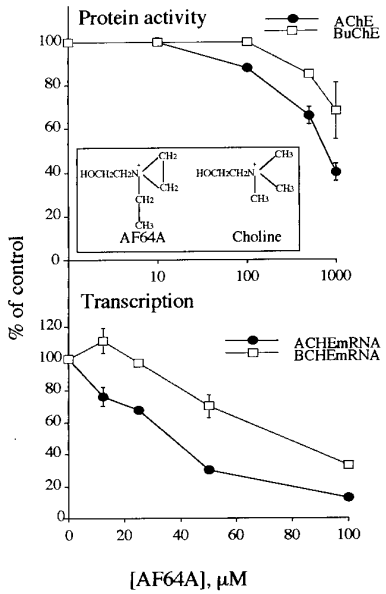


Fig. 2. In vitro effects of AF64A on mammalian CHE genes and their enzyme products. Top: direct AF64A induced inhibition of cholinesterase activities. Inhibition was measured following incubation with increasing concentrations of AF64A by determining remaining activities of AChE and BChE, all as detailed under methods. Inset: chemical structures of the cholinotoxin AF64A (a) and the native choline molecule (b), after Fisher et al. [6]. Bottom: differential sensitivity of the AChE and BChE genes for transcriptional damage induced by AF64A. Equal amounts of the noted plasmids incubated with the noted concentrations of AF64A were used for in vitro transcription in the presence of [^{32}P] nucleotides followed by agarose gel electrophoresis and autoradiogrephy of the ^{32}P-labeled reaction products. Note the relative resistance to AF64A of BChE – as compared with AChEcDNA. The same results were obtained for BChE mRNA transcribed from two transcription plasmids containing the human BChE coding sequences with two distinct RNA polymerases (not shown), demonstrating that the AF64A effects were due to the cDNA seqence and not to the type of RNA polymerase.

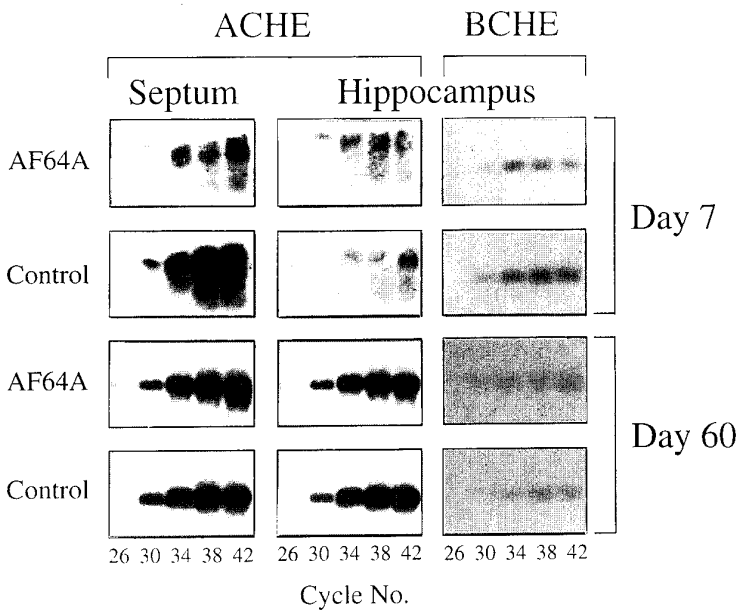


Fig. 3. In vivo modifications in cholinesterase mRNA levels following AF64A treatment. RNA was extracted from septum, hippocampus and striatum of 3 pooled animals 7 and 60 days post AF64A treatment and was subjected to RT-PCR procedure [17]. PCR products were detected as dark bands after hybridization followed by autoradiography.

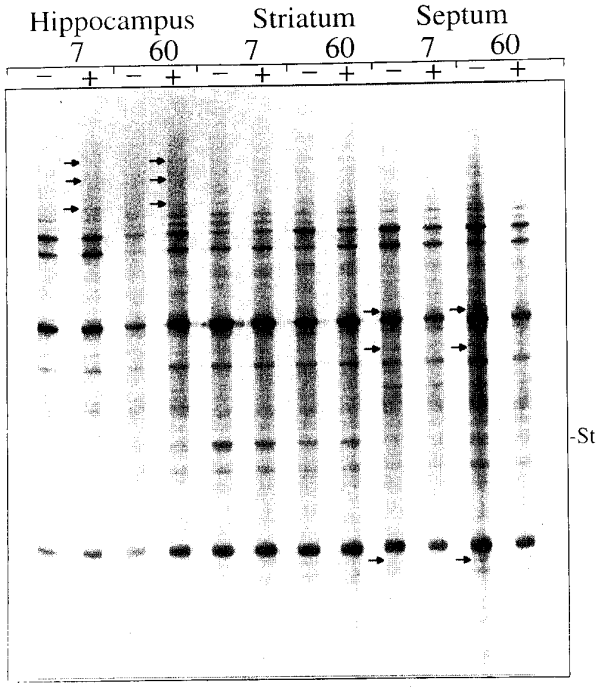


Fig. 4. PCR display. Differential PCR display of 1 μg total RNA extracted from hippocampus, striatum and septum pooled regions (from 3 animals/sample) of saline (–) and AF64A (+) injected rat brains, 7 and 60 days post treatment. Exposure was for 1 day at room temperature, for 10% of reaction mixture per lane. PCR fragments size was between 200 and 400 bp. Altogether most of the PCR products were common to the different brain regions; some were specific for either striatum (St) or hippocampus (not shown in this part of the gel). Arrows indicate PCR products whose intensity changed following AF64A injection.

Conclusions

In conducting this research, we employed transgenic *Xenopus* tadpoles to reveal the molecular mechanisms leading to the accumulation of AChE in synapses. We have found that the 40 amino acid-long C-terminal peptide of the brain form of AChE is an essential requirement for this accumulation. In contrast, the 42 amino acids of the "readthrough" form of this enzyme, which so far has been demonstrated only at the level of the ACHE gene and its mRNA products, supports epidermal expression and excretion of a more hydrophilic monomeric form of this enzyme.

The *Xenopus* data implied that to obtain synaptic protection against OP poisons, one needs the brain cDNA. Therefore, we constructed transgenic mice expressing such DNA in their CNS neurons. These mice indeed became resistant to the hypothermia-inducing effects of OPs. However, they also developed progressive deterioration of learning and memory, suggesting that for safer protection one needs to confer expression of the transgenic enzyme in tissues other than brain.

OP and carbamate interactions of AChE and BuChE and their natural variants were studied in *Xenopus* oocyte-produced recombinant proteins, following their partial purification and enrichment onto selective monoclonal antibodies in microtiter plates. These tests demonstrated that BuChE interacts faster and more efficiently than AChE with a number of these anti-ChEs, suggesting that it operates *in vivo* as a scavenger of these inhibitors and drugs of these categories.

Interestingly, the relatively common "atypical" allelic variant of BuChE was found to differ from its normal counterpart by being incapable of scavenging carbamate and quaternary amine inhibitors, including pyridostigmine and tacrine. This, in turn, implies that in individuals carrying this allelic variant, the effective dose of anti-ChEs would be higher than in others, which may lead to adverse responses to such drugs.

To test the effects of cholinotoxic damage in the mammalian brain, we adapted the use of differential PCR display to dissected brain regions. Using this method, we demonstrated long-lasting (months long) changes in gene expression in brain regions of rats subjected to mild intoxication of cholinergic neurons.

Altogether, the studies performed in this first year of our research advanced our knowledge regarding the biology of cholinergic mechanisms in the mammalian brain and the involvement of ChEs in these important processes.

References

Anglister L and McMahan UJ (1985) *J. Cell Biol.* 101:735-743.

Beeri R, Gnatt A, Lapidot-Lifson Y, Ginzberg D, Shani M, Soreq H and Zakut H (1994) *Human Reprod.* 9:284-292.

Carmichael M (1994) *Sci. Am.* 270:64-72.

Chrobak JJ, Hanin I, Schmechel DE and Walsh TJ (1988) *Brain Res.* 4632:107-117.

Cohen MW (1980) J. Exp. Biol. 89:43-56.

Ehrlich G, Ginzberg D, Loewenstein Y, Glick D, Kerem B, Ben-Ari S, Zakut H and Soreq H (1994) *Genomics* 22:288-245.

Eichenbaum H, Steward C and Morris RGM (1990) *J. Neurosci.* 10: 3531-3542.

Enz A, Amstutz R, Boddeke H, Gmelin G, Malonowski J (1993) *Brain Res.* 98-431-437.

Fisher A, Manitone CR, Abraham DJ and Hanin I (1982) *J. Pharm. Exp. Ther.* 222: 140-145.

Flucher BE and Daniels MD (1989) *Neuron* 3:163-175.

Froehner SC (1991) *J. Cell Biol.* 114:1-7.

Futscher BW, Pieper O, Barnes DM, Hanin I and Erickson LC (1992) *J. Neurochem.* 58:1504-1509.

Giacobini E (1991) In R Becker and E Giacobini (eds.) *Cholinergic Basis for Alzheimer Therapy*, Birkhauser, Boston , 247-262.

Gilson MK, Straatsma TP, McCammon JA, Ripoll DR, Faerman CH, Axelsen PH, Silman I and Sussman JL (1994) *Science* 263:1276-1278.

Gindi T and Knowland J (1979) *Exp. Morphol.* 51:209-215.

Hanin I, Yaron, A, Ginzberg D and Soreq H (1994). In I Hanin, A Fisher and M Yoshida (eds.) *Alzheimer's and Parkinson's Diseases:Recent Advances*, Plenum Press, New York.

Iversen LL (1993) *Brain Res.* 98:423-426.

Kalow W and Davis RO (1958) *Biochem. Pharmacol.* 1:183-192.

Karnovsky MJ (1964) *J. Cell Biol.* 23:217-232.

Karpel R, Ben Aziz-Aloya R, Sternfeld M, Ehrlich G, Ginzberg D, Tarroni P, Clementi F, Zakut H and Soreq H (1994) *Exp. Cell Res.* 210:268-277.

Keeler JR, Hurst CG, Dunn MA (1991) *J. Am. Med. Assn.* 266:6903-695.

Kitt CA, Höhmann C, Coyle JT and Price DL (1994) *J. Comp. Neurol.* 341:117-129.

Knapp MJ, Knopman DS, Solomon PR, Pendlebury WW, Davis CS, Gracon SI (1994) *J. Am. Med. Assn.* 271:985-991.

Kothary R, Barton SC, Franz T, Norris ML, Hettle S and Surani AMH (1991) *Mech. Develop.* 35: 25-31.

Kronman C, Velan B, Gozes Y, Leitner M, Flashner Y, Lazar A, Marcus D, Sery T, Papier A, Grosfeld H, Cohen S and Shafferman A (1992) *Gene* 121:295-304.

Kullberg RW, Lentz TL and Cohen MW (1977) *Devel. Biol.* 60: 101-129.

Kullberg RW, Mikelberg FS and Cohen MW (1980) *Devel. Biol.* 75:255-267.

Leventer SM, Wulfert E and Hanin I (1987) *Neuropharmacol.* 26:361-365.

Liang P and Pardee AB (1992) *Science* 257:967-971.

Liao J. Heider H, Sun MC and Brodbeck U (1992) *J. Neurochem.* 58:1230-1238.

Liao J, Mortensen V, Nørgaard-Pedersen B, Koch C and Brodbeck U (1993) *Eur. J. Biochem.* 215:333-340.

Lockridge O (1990) *Pharmacol. Ther.* 47:35-60.

Loewenstein Y, Gnatt A, Neville LF and Soreq H (1993) *J. Mol. Biol.* 234:289-296.

Manitone CR, Fisher A and Hanin I (1981) *Science* 213:579-580.

McGuire MC, Nogueira CP, Bartels CF, Lightstone H, Hajra A, van der Spek AFL, Lockridge O and La Du BN (1989) *Proc. Natl. Acad. Sci USA* 86:953-957.

Morris RGM, Garrud P, Rawlins JNP and O'Keefe J (1981) *Nature* 297: 681-682.

Neville LF, Gnatt A, Loewenstein Y and Soreq H (1990) *J. Neurosci Res.* 27:452-460.
Neville LF, Gnatt, Loewenstein Y, Seidman S, Ehrlich G and Soreq H (1992) *EMBO J.* 11:1641-1649.
Ohlendieck K, Ervasti JM, Matsumura K, Kahl SD, Levelille CJ and Campbell KP (1991) *Neuron* 7:499-508.
Ordentlich A, Barak D, Kronman C, Flashner Y, Leitner M, Ariel N, Cohen S, Velan B and Shafferman A (1993) *J. Biol. Chem.* 268:17083-17095.
Prody CA, Zevin-Sonkin D, Gnatt A, Goldberg O and Soreq H (1987) *Proc. Natl. Acad. Sci USA* 84:3555-3559.
Quinn D (1987) Chem. Rev. 87:955-979.
Raveh L, Grunwald J, Marcus D, Papier Y, Cohen E and Ashani Y (1993) *Biochem. Pharmacol.* 45:2465-2474.
Rosenberry TL (1975) *Adv. Enzymol.* 43:1-4-210.
Sandberg K, Hanin I, Fisher A and Coyle JT (1984) *Brain Res.* 293:49-55.
Schwarz M, Glick D, Loewenstein Y and Soreq H (1995a) *Pharmacol. Therap.* (in press).
Schwarz M, Loewenstein-Lichtenstein Y, Glick D, Liao J, Nørgaard-Pedersen B and Soreq H (1995b) *Mol. Brain Res.* (in press).
Seidman S, Ben Aziz-Aloya R, Timberg R, Loewenstein Y, Velan B, Shafferman A, Liao J, Nørgaard-Pedersen B and Soreq H (1994) *J. Neurochem.* 62:1670-1681.
Seidman S, Sternfeld M, Ben Aziz-Aloya R, Timberg R, Kaufer-Nachum D, Soreq H (1995) *Mol. Cell. Biol.* (in press).
Shafferman A, Velan B, Ordentlich A, Kornman C, Grosfeld H, Leitner M, Flashner Y, Cohen S, Barak D and Ariel N (1992) *EMBO J.* 11:3561-3568.
Shapira M, Seidman, S, Sternfeld M. Timberg R, Kaufer D, Patrick J and Soreq H (1994) *Proc. Natl. Acad. Sci. USA* 91:9072-9076.
Simpson CV, Ruwe WD and Myers RD (1994) *Neurosci. Behavior. Rev.* 18:1-20.
Soreq H and Zakut H (1993) *Human Cholinesterases and Anticholinesterases*, Academic Press, San Diego.
Soreq H, Ben Aziz-Aloya R, Prody C, Seidman S, Gnatt A, Neville L, Lieman-Hurwitz J, Lev-Lehman E, Ginzberg D, Lapidot-Lifson Y and Zakut H (1990) *Proc. Natl. Acad. Sci. USA* 87:9688-9692.
Taylor P (1990) In LS Goodman, AG Gilman, TW Rall, AS Nies and P Taylor (eds.) *Pharmacological Basis of Therapeutics*, Macmillan Publishing Co., New York, 131-147.
Velan B, Grosfeld H, Kronman C, Leitner M, Gozes Y, Lazar A, Flashner Y, Marcus D, Cohen S and Sharfferman A (1991a) *J. Biol. Chem.* 266:23977-23984.
Velan B, Kronman C, Grosfeld H, Lietner M, Gozes Y, Flashner Y, Sery T, Cohen S, Ben-Aziz R, S Seidman S, Shafferman A and Soreq H (1991b) *Cell. Mol. Neurobiol.* 11:143-156.
Vellom DC, Radic Z, Li Y, Pickering NA, Camp S and Taylor P (1993) *Biochemistry* 32:12-17.
Welsh J, Chada K, Dalal S, Cheng R, Ralph D and McClelland M (1992) Nuc. Acid Res. 20: 4965-4970.
Whittaker M (1986) *Cholinesterase*, Karger, Basel.
Willems JL, DeBisschop HC, Verstraete AG, Declerck C, Christiaens Y, Vanscheeuwyck P, Buylaert WA, Vogelaers D and Colardyn F (1993) *Arch. Toxicol.* 67:79-84.
Wilson IB (1954) In WD McElroy and B Glass (eds.) *The Mechanism of Enzyme Catalysis,* Johns Hopkins Press, Baltimore.
Winker MA (1994) *J. Am. Med. Assn.* 271:1023-1024.
Wurtman RJ (1992) *Trends Neurosci.* 15:117-122.
Yu J, Thomson R, Huestis PW, Bjelajac VM and Crinella FM (1989) *Physiol. Behav.* 45:133-144.

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Testicular amplification and impaired transmission of human butyrylcholinesterase cDNA in transgenic mice

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Gene amplification occurs frequently in tumour tissues yet is, in general, non-inheritable. To study the molecular mechanisms conferring this restraint, we created transgenic mice carrying a human butyrylcholinesterase (BCHE) coding sequence, previously found to be amplified in a father and son. Blot hybridization of tail DNA samples revealed somatic transgene amplifications with variable restriction patterns and intensities, suggesting the occurrence of independent amplification events, in 31% (11/35) of mice from the FII generation but in only 3.5% (2/58) of the FIII and FIV generations. In contrast, >10-fold amplifications of the BCHE transgene and the endogenous acetylcholinesterase and c-*raf* genes appeared in both testis and epididymis DNA from >80% of FIII mice. Drastic, selective reductions in testis BCHEmRNA but not in actin mRNA were detected by the PCR amplification of testis cDNA from the transgenic mice, and apparently resulted in the limited transmission of amplified genes. The testicular amplification of the BCHE transgene may potentially represent a general phenomenon with clinical implications in human infertility.

Key words: cholinesterase/fertility/human/polymerase chain reaction/testicular gene amplification

Introduction

Gene amplification is common in eukaryotic chromosomes of tumour tissues and transfected cells (Delidakis *et al.*, 1989; Stark *et al.*, 1989). Amplification often provides cells in which it occurs with resistance to toxic ligands which bind to the over-expressed protein products of the amplified genes (Schimke, 1990). Alternatively, over-expressed proteins may support proliferation as in the case of oncogene products (Bishop, 1991) or, as found in amphibia and insects, they may be necessary for essential processes during development (Laat *et al.*, 1986). The inheritance of amplified sequences at a specific gene locus has been assessed in the present work.

Both novel point mutations (Cooper and Schmidtke, 1987) and the expansion of CGG and CTG repeats within the fragile X (Oberle *et al.*, 1991; Kremer *et al.*, 1991) and the myotonic dystrophy domains (Brook *et al.*, 1992; Harley *et al.*, 1992), are successfully transmitted to subsequent generations. In contrast, gene amplifications are, in general, non-inheritable. One exception is the heritable esterase gene amplification which provides resistance to insecticides in mosquitoes (Mouches *et al.*, 1986). In humans, the BCHE gene encoding butyrylcholinesterase (acylcholine acyl hydrolase, BCHE, EC. 3.1.1.8) was observed to be amplified in a father and son exposed to the Pro-insecticide *N*-methyl parathion, which functions through cholinesterase inhibition (Prody *et al.*, 1989). However, there was some doubt as to whether the amplified DNA was actually inherited, or whether a similar propensity for amplification in two genetically similar individuals was inherited. Subsequently, the BCHE gene, together with the related ACHE gene encoding acetylcholinesterase (acetylcholine acetyl hydrolase, ACHE, EC, 3.1.1.7) and several oncogenes were found to amplify in leukaemias (Lapidot-Lifson *et al.*, 1989) and ovarian tumours (Zakut *et al.*, 1990) and in non-cancerous disorders of haemopoietic development, such as the impaired megakaryocytopoiesis in the autoimmune disease lupus erythematosus (Zakut *et al.*, 1992). These observations make the BCHE gene particularly attractive for studies of the amplification phenomenon. Here, we describe a transgenic model for a germline BCHE gene amplification, and the developmental mechanism by which this potentially stable amplification event was selectively precluded from the pool of inherited genetic material.

Material and methods

Mouse strains and microinjection

The pSVL-CHE sequence (see Figure 1 for details) was employed for microinjection into fertilized mouse eggs which had been flushed from the oviduct of (C57BL/6J × BALB/C) FI females mated with (C57BL/6J × DBA) FI males. Manipulations of the mice and eggs, and the microinjection techniques, were as previously described (Shani, 1985).

Probes

Purified, electro-eluted probes included a 1.5 kb long *Eco*RI fragment of ACHEcDNA (Lapidot-Lifson *et al.*, 1989) and a

2.4 kb long *Pst*I−*Sac*I fragment of BCHEcDNA (Prody *et al.*, 1987). C-*raf* DNA was obtained from Amersham (Buckinghamshire, UK). SV-40 DNA was gratefully received from Professor S.Lavi (Tel-Aviv), haptoglobin cDNA-from Dr E.Zerial (Heidelberg) and the 1.4 kb *Eco*RI repetitive DNA fragment was eluted from a gel following electrophoresis of total mouse genomic DNA after digestion with *Eco*RI. All probes were labelled with [^{32}P]dATP by the random primed technique using the relevant kit from Boehringer (Mannheim, Germany).

Quantification of amplification by slot blot hybridization

Denatured genomic DNA from tail, testis or epididymis was diluted and spotted onto Gene Screen filters (NEN, Boston, MA, USA). Slot blot hybridization and wash stringency were as previously detailed (Prody *et al.*, 1989), using genomic or electro-eluted insert DNA supplemented with denatured herring testis DNA to yield a total of 2 μg DNA per slot. Signals were totally abolished following DNAse treatment but were unaffected by 0.4 N NaOH, excluding the possibility of non-specific binding due to RNA contamination. Exposure was carried out for 3 days at −70°C with an intensifying screen. DNA copy numbers were calculated as described (Lapidot-Lifson *et al.*, 1989) by using densitometric comparisons to the signals obtained with serial dilutions of the purified cDNA.

DNA blot hybridization

Genomic DNA samples digested with restriction enzymes were electrophoresed on 1.0% agarose gels, transferred to filters and subjected to hybridization under the same conditions used for the slot blot analyses. Lambda and QX174 phage DNAs cut with *Hin*dIII served for molecular weight markers. Exposure was carried out overnight.

Evaluation of sperm motility

Mouse spermatozoa extruded from dissected epididymis were placed in 50 μl drops on microscope slides and motility evaluated at 23°C, essentially as detailed elsewhere (Makler, 1991).

Results

BCHE coding sequences amplify in transgenic mice

Three out of 17 founder transgenic mice created as previously detailed (Shani, 1985) with the BCHE coding sequence, the late SV-40 promoter and late polyadenylation site (pSVL-CHE DNA, Figure 1) were shown by tail DNA hybridization to carry a single transgenic sequence hybridizing with BCHEcDNA. Two of these founders were mated with C57B1/6J mice. The FI offspring examined still carried single copies of BCHE DNA.

Two independent FII pedigrees were subsequently established (nos 12 and 40). Slot blot hybridization tail DNA demonstrated the appearance of gene amplification characterized by a wide variability in BCHE DNA copy number among FII mice (Figure 2). All mice appeared to be healthy, and no tumours could be detected in any of their tissues. Altogether 31% of the tail DNA samples from FII mice carried multiple (5−200) copies of the BCHE DNA sequence (Figure 3).

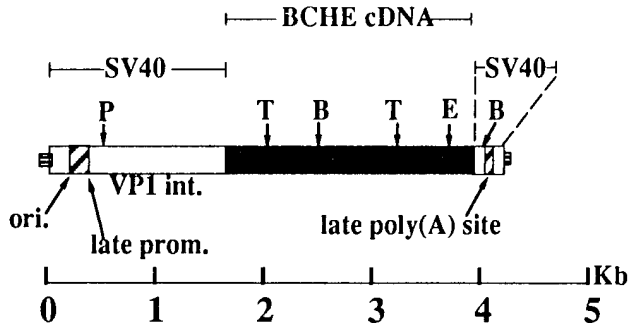

Fig. 1. The pSVL-CHE DNA construct. The 4.7 kb pSVL-CHE construct was created by ligating the 2.3 kb long human BCHE cDNA insert (Prody *et al.*, 1987), bordered by *Pst*I and *Sac*I sites, into the pSVL plasmid (Pharmacia, Sweden) constructed with parts of pBR and the SV-40 genome. The 7.2 kb circular DNA product was cut with the enzymes *Aht*II and *Nar*I to yield the linear pSVL-CHE transgene which includes CHE cDNA with upstream SV-40 origin of replication (ori.), late promoter (prom.) and VP-1 intron (int.), and with the SV-40 late polyadenylation site downstream. Short (30−70 bp) sequences from the pBR plasmid remained at both ends of this construct (hatched boxes). Restriction sites for the enzymes *Taq*I (T), *Bam*HI (B), *Pvu*II (P) and *Eco*RI (E) are noted.

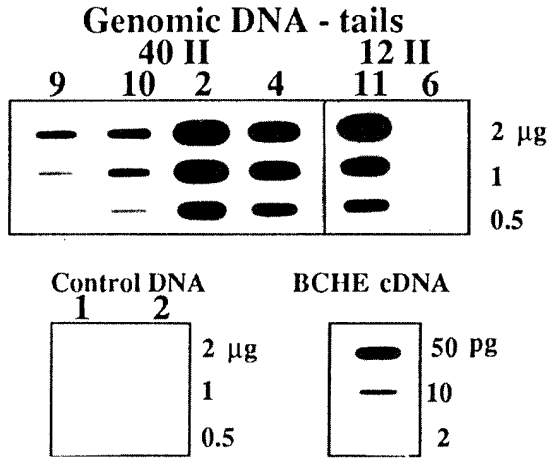

Fig. 2. Quantification of BCHE DNA amplification levels in tail DNA samples. The autoradiogram of a slot blot DNA hybridization experiment with tail DNA from six representative transgenic mice (40 II-2, 4, 9, 10 and 12 II-6, 11) and two control non-transgenic mice is shown. DNA quantities are noted. BCHE sequence copy numbers were calculated (Lapidot-Lifson *et al.*, 1989) to be ~20 and 100 for mice 40 II-10 and 12 II-11, respectively.

Variable litter sizes

Litter sizes were highly variable in the transgenic mice, with a range of 3−13 mice per litter within 11 such litters of the FI−FIV generations (Figure 3). For comparison with other transgenic mice having no DNA amplification, we recently observed a far less variable range of 10−12 mice per litter among seven litters. Thus, the mere introduction of an exogenous transgene was not sufficient to induce this variability in litter sizes. This, in turn, suggests that the amplification phenomenon may be correlated with reduced fertility. However, because of these small sample sizes, further analyses will be required to establish statistically significant causal relationships between the observed

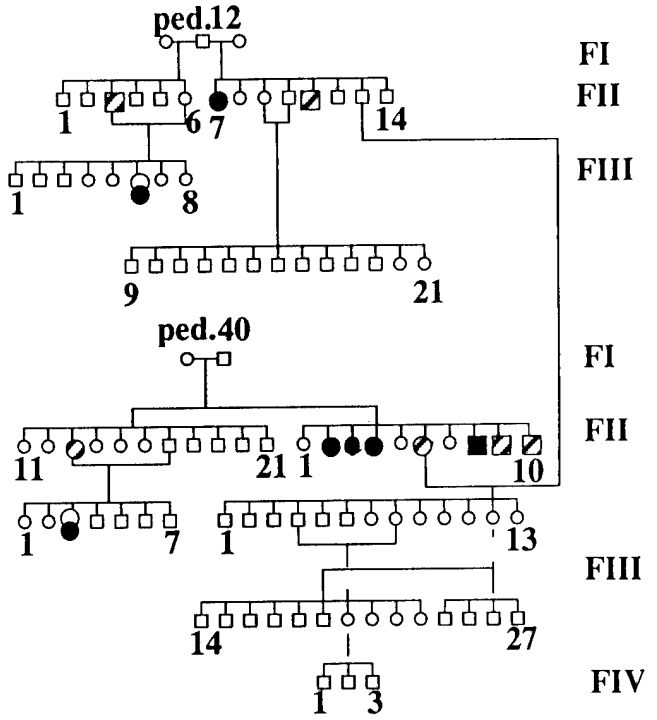

Fig. 3. Somatic amplification of CHE coding sequences in pSVL-CHE pedigrees. Tail DNA from four generations of the pSVL-CHE pedigrees 12 and 40 was examined for copy numbers of DNA sequences from the coding regions of the BCHE and ACHE genes. Generations (F) are noted by roman numerals. Squares = males, circles = females. Dashed and black signs = >20 and 100 copies of BCHE DNA, respectively. Double circle signs = co-amplified ACHE DNA, >100 copies. Open symbols = non-transgenic and low copy transgenic mice.

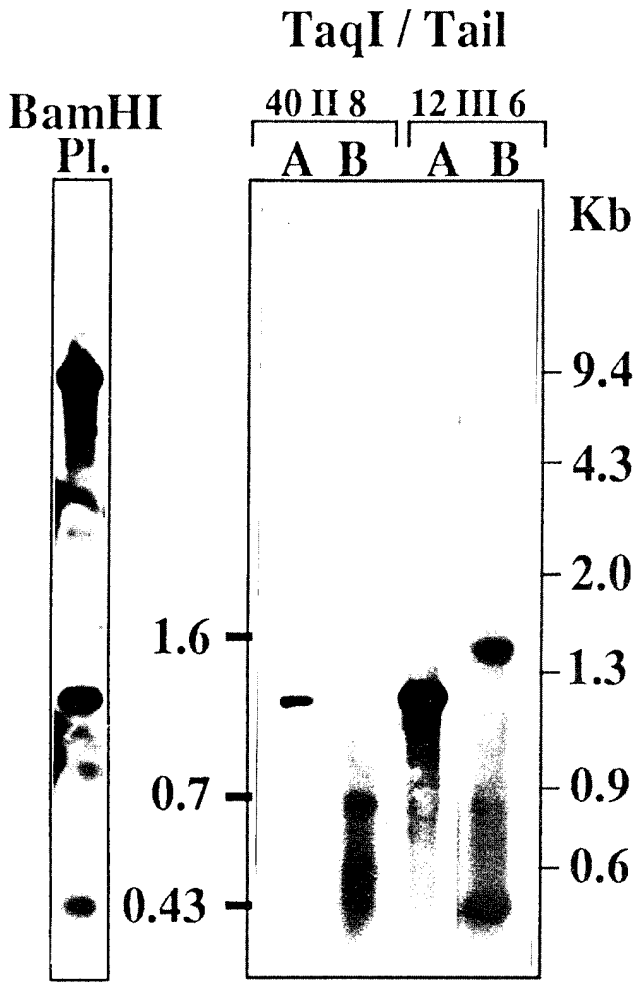

Fig. 4. Restriction fragment analysis of somatic ACHE and BCHE DNA. **Right**: 10 μg tail DNA samples from mice 40 II-8 and 12 III-6 were digested with *Taq*I, electrophoresed and subjected to DNA blot hybridization with BCHE (B) and then with ACHEcDNA (A) probes, followed by autoradiography. **Left**: 1 ng plasmid DNA which contains the human ACHE DNA was cut by *Bam*HI, electrophoresed and hybridized with the 1.5 kb ACHE cDNA probe, to reveal hybridization standard equivalent to 100 copies of the ACHE sequence.

DNA amplification and the apparently reduced fertility in these transgenic mice.

Limited transmission of the amplification and appearance of amplified ACHE gene

Only 3.6% of FIII generation mice (2/55) in six different litters displayed somatically amplified BCHE DNA sequences (Figure 3). Blot hybridization revealed major *Taq*I-digested BCHE DNA fragments of different sizes and confirmed the amplification levels in tail DNA from various mice (Figure 4). Since BCHE coding sequences co-amplify with the ACHE coding region in leukaemias (Lapidot-Lifson *et al.*, 1989), ovarian carcinomas (Zakut *et al.*, 1990) and lupus erythematosus (Zakut *et al.*, 1992), the endogenous ACHE DNA coding sequence was also examined in the pSVL CHE transgenic mice. None of the FII mice displayed somatic ACHE DNA amplification. In contrast, tail DNA from the two FIII mice carrying amplified BCHE DNA, but not other FIII mice, also carried co-amplified ACHE DNA (Figures 3 and 4). A unique *Taq*I restriction pattern was observed for the endogenous amplified DNA which displayed short DNA fragments hybridizing with the G,C-rich human [^{32}P]ACHEcDNA (Soreq *et al.*, 1990), easily distinguishable from the pattern derived from the BCHE probe (Figure 4).

Characterization of somatically amplified transgenic BCHE DNA

Variability in intensities and length of the amplified restriction fragment were observed for BCHE DNA fragments in different members of single FII litters (Figure 5A), suggesting that indepenent amplification events had occurred. Amplified BCHE DNA generally presented major BamHI fragments, possibly due to repeated arrangements of the amplification units. These fragments, sized between 4.2 and 6.5 kb each, were all larger than those expected to be derived from the transgene. Rehybridization with SV-40 DNA revealed fragments having the same sizes as the major BCHE DNA-labelled fragments (Figure 5B). The strong hybridization signal with the viral SV-40 probe implied that the amplified sequence was of exogenous origin. In addition, the non-similar intensities of signals with the BCHE and SV-40 probes imply, at least in one case, variable copy numbers of the corresponding regions in the transgene.

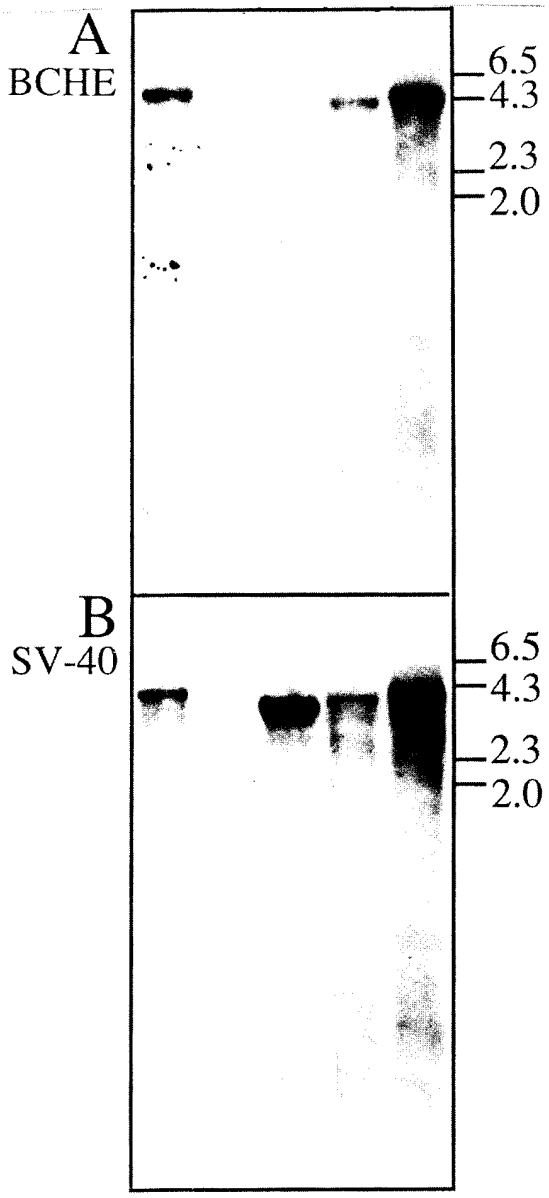

Fig. 5. *Bam*HI analysis of BCHE and SV-40 amplified sequences in tail DNA. 10 μg tail DNA samples of five females, all belonging to a single 40 FII litter were digested by *Bam*HI and subjected to blot hybridization with the BCHE cDNA probe (**A**) and the SV-40 probe (**B**). Exposure: **A** overnight, **B** over 2 days. Samples, in the following order from left to right, were derived from mice 3, 5, 6, 4 and 2 (Ped 40, FII generation). These DNA samples plus additional controls were further subjected to hybridization with *Eco*RI repetitive DNA fragment which revealed similar, multiband restriction patterns and similar labelling intensities with all the examined DNA samples (not shown), implying that the amplified sequences were limited in size and that no gross changes occurred in the host genome.

Part of the restriction sites included in the original transgene were lost in the amplified pSVL-CHE sequences [i.e. a *Bam*HI site missing in pedigree 40 FII DNAs; (Figure 5A,B)]. This could reflect rearrangements within the amplification unit, a common event in other amplified genes (Libermann *et al.*, 1985).

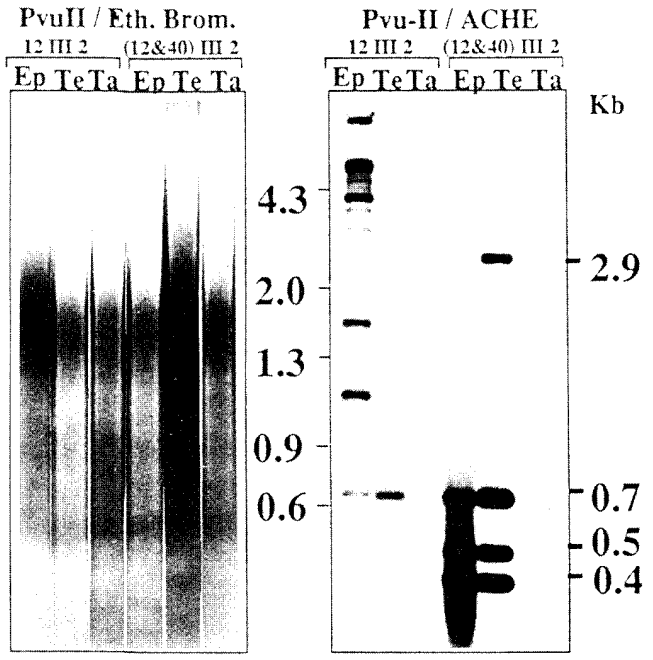

Fig. 6. Restriction fragment analysis of amplified ACHE DNA in testis and epididymis of pSVL-CHE transgenic mice. 10 μg DNA samples from tail (Ta), testis (Te) and epididymis (Ep) of mice 12 III-2 and 12/40 III-2 were digested with the restriction enzyme *Pvu*II, electrophoresed and subjected to DNA blot hybridization with the ACHE cDNA probe followed by autoradiography (**right**). Ethidium bromide staining of the digested samples (**left**) demonstrates similar amounts of DNA in all lanes. Digestion of EpDNA from sample 12 FII-2 was apparently incomplete.

Amplification of CHE genes and oncogenes in testis and epididymis

To monitor the fate of the amplified DNA between generations, we hybridized DNA from somatic and germline tissues with cDNA probes for ACHE, BCHE, the ubiquitous amplifiable oncogene c-*raf*, and the tissue-specific haptoglobin gene. Whereas neither tail (Figures 6 and 7) nor liver DNA (not shown) from any of the selected FII or FIII mice carried somatically amplified genes, all of these mice displayed BCHE, c-*raf* and SV-40 amplifications in DNA from testis and epididymis (Figure 7 and data not shown). Most of these samples also carried amplified ACHE DNA (Figures 6 and 7), and c-*fes*(*fps*) DNA (not shown). The extent of gene amplification observed with the various probes appeared similar within particular mice. In contrast, haptoglobin DNA was found in equally low copy numbers (1−3) in all tissues examined (Figure 7). The *Pvu*II restriction pattern observed for the amplified endogenous ACHE gene in the testis and epididymis of the transgenic mice (Figure 6) confirmed the enhancement in hybridization signals observed by DNA slot hybridization and resembled, in all respects, the pattern observed previously for this gene when amplified in human peripheral blood cells (Lapidot-Lifson *et al.*, 1989; Zakut *et al.*, 1992).

DNA blot hybridization following *Eco*RI and *Pvu*II digestions of epididymis and testis DNA revealed labelled fragments of similar sizes using SV-40 and BCHE cDNA probes (Figure 8). This observation demonstrated their transgenic origin, in a manner similar to the somatic amplifications. The observed single

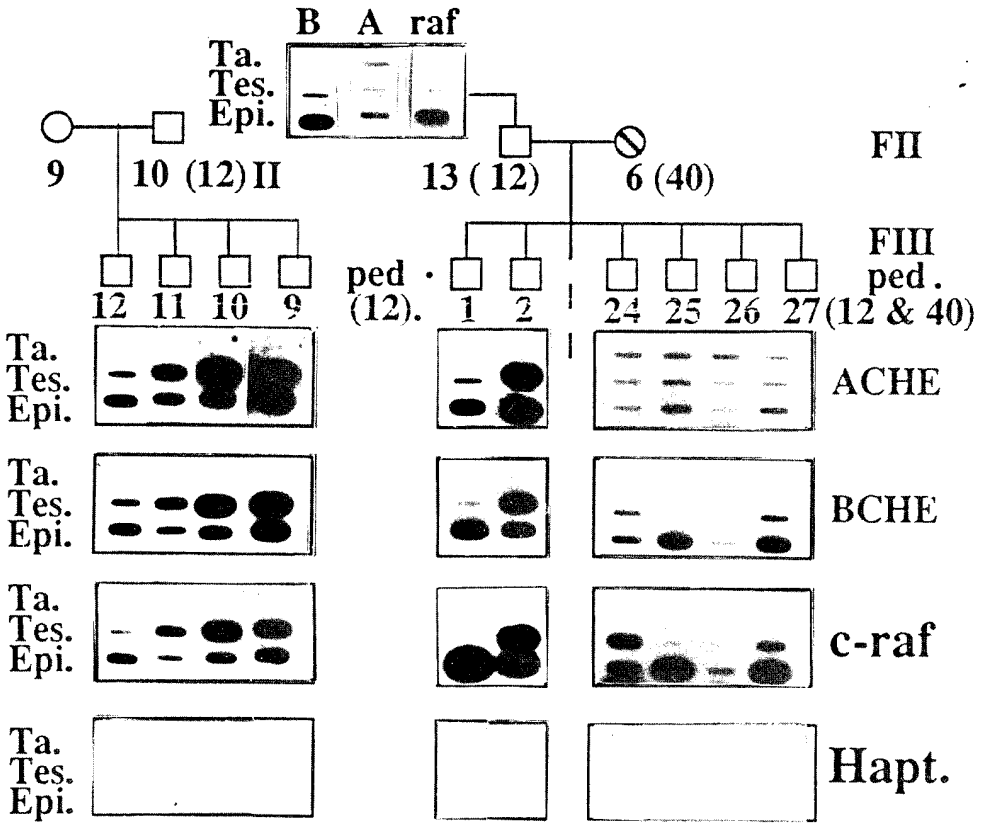

Fig. 7. Selective amplification of ACHE, BCHE and c-*raf* DNA in testis and epididymis of transgenic mice. Hybridization with the ACHE, BCHE, c-*raf* and haptoglobin (Hapt.) DNA probes were performed (as in Figure 2) with tail, testis and epididymis DNA from one adult FII male and 10 FIII males. These were 3 months old (no. 9–12, pedigree 12 and no. 1, 2, pedigree 12/40) or 4 weeks old (no. 24–27, pedigree 12/40). Dashed circles: ~20 copies of BCHE DNA in tail DNA. Five non-transgenic control mice were found to carry single copies of each of the analysed sequences in all tissues (not shown). Quantification was done as described (Lapidot-Lifson *et al.*, 1989).

fragments, however, appeared to be shorter than the complete transgene (Figure 8). This suggested the occurrence of deletions within the rearranged, amplified testicular transgenic sequences.

Selective BCHEmRNA reductions in testis of transgenic mice

To search for putative correlations between the testicular gene amplifications and spermatogenesis, developmental alterations were pursued. There were no clear differences in BCHE, ACHE and c-*raf* copy numbers between testis and epididymis DNA from adult mice. However, in 4-week-old mice the hybridization signals with DNA from testis, which at this age is poorer in mature sperm cells, were significantly lower than those observed with epididymis DNA (Figure 7). This observation potentially correlated the amplification phenomenon with sperm cell maturation. Interestingly, similar increases occur in copy numbers of the CGG and CTG repeats in the fragile X and myotonic dystrophy genes during germ cell development (Oberle *et al.*, 1991; Kremer *et al.*, 1991; Brook *et al.*, 1992; Harley *et al.*, 1992). The loss of amplification from subsequent generations could hence be due to the defective properties of germ cells in which the amplification occurred.

Further experiments were initiated to examine transcription in testis tissue from the transgenic mice. Gene expression in mammalian spermatogenic tissue is considered to be confined to the limited number of transcripts essential for germ cell development and/or function (Richler *et al.*, 1992; Willison and Ashworth, 1987). Cholinesterase and choline acetyltransferase activities were observed in mammalian sperm cells (Rama Sastry and Sadavongvivad, 1979; Rama Sastry *et al.*, 1981), and several reports have implicated acetylcholine with sperm motility (Ibanez *et al.*, 1991). The presence of BCHEmRNA transcripts was therefore examined in testis of control and transgenic mice by the use of direct reverse transcription coupled with PCR amplification (RNA–PCR). Parallel RNA–PCR amplification was performed with primers from the mouse gene encoding smooth muscle γ-actin (SMGA), which is one of the few transcripts known to be expressed during spermiogenesis (Kim *et al.*, 1989).

Total RNA from testis of two control mice and six transgenic mice carrying between five and 15 copies of the amplified genes in their testis DNA displayed apparently similar levels of actin mRNA (Figure 9). In addition, testis smears from adult transgenic mice showed normal density and a regular morphology of sperm cells. An apparently normal motility was observed in sperm cells extruded from epididymal preparations of these mice. In contrast, the results of RNA–PCR using BCHE primers reflected drastic reductions in BCHEmRNA levels in the testis of transgenic mice carrying pSVL-CHE amplifications versus control mice (Figure 10). In two of the transgenic mice, the BCHE PCR product could not be observed at all. This result implies that testis RNA from

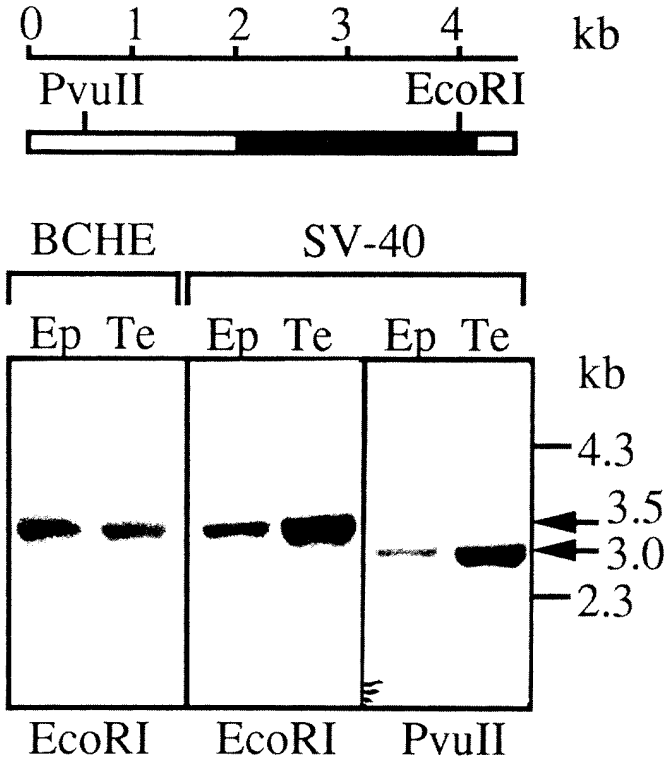

Fig. 8. *Eco*RI and *Pvu*II analysis of BCHE and SV-40 amplified sequences in testis and epididymis DNA. 10 μg testis (Te) and epididymis (Ep) DNA samples from mice 12/40 FIII nos 2 and 1, respectively (see Figure 7), were cut separately by *Eco*RI and *Pvu*II and were hybridized with BCHE and SV-40 probes. Restriction sites for *Pvu*II and *Eco*RI within the pSVL-CHE are shown above. Rehybridization with isolated 300 bp long SV-40 origin probe demonstrated that this sequence is included in the amplified DNA, indicating a tandem organization.

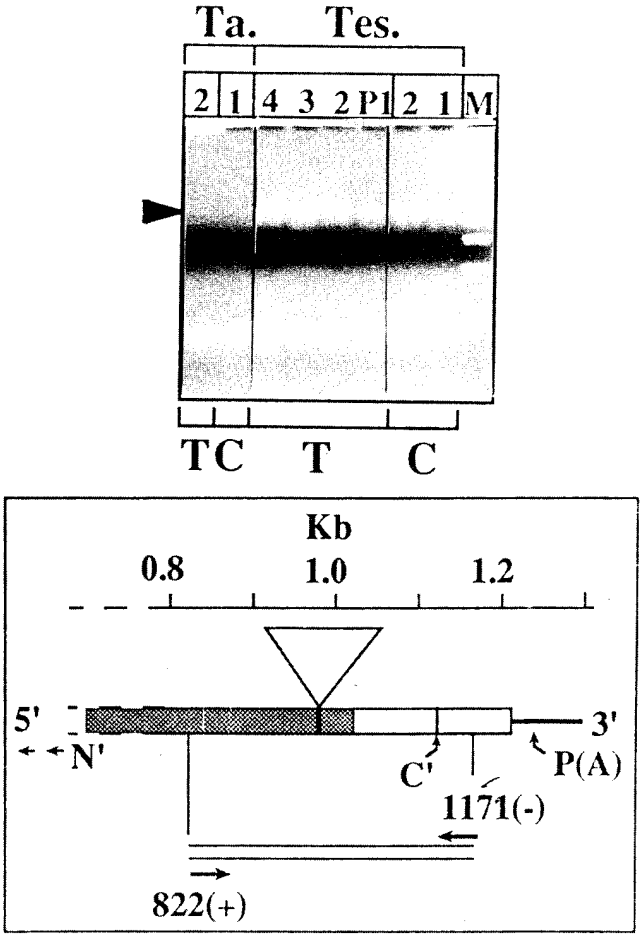

Fig. 9. Maintenance of actin mRNA levels in testis of transgenic mice. Testis (Tes) and tail (Ta) RNA was extracted from control (C) mice and from six adult transgenic (T) mice: 2, 3, 4 = 12/40 FIV-1, 2, 3, P1 = pooled RNA from mice 12 FIII-11, 12 and 12/40 FIII-1 (Figure 3). Reverse transcription was performed using the RNA–PCR kit (Perkin Elmer Cetus, Norwalk, CT, USA) following the manufacturer's recommendations, except that incubation with MuLV reverse transcriptase was extended for 25 min. PCR amplification of the resultant Actin cDNA (35 cycles) was performed using oligodeoxynucleotide primer pairs from two different exons in the mouse smooth muscle actin γ-actin (SMGA) (Kim *et al.*, 1989), primers mACT 822(+), 5'TGAAACAACATA-CAATTCCATCATGAAGTGTGAC-3' and 1171(−), 5'-TGGCT-GGTGACCAAGTCTTGTGGGGAT-3'. See Nakqjima-Iijima *et al.* (1985) and Veyama *et al.* (1984) for details on actin gene structure. Upstream and downstream orientations are noted by (+) and (−) signs, respectively. Numbers indicate the 5' end position in each of these primers within the relevant cDNA sequence. The positions of the primers within different exons are shown (down). RNA–PCR products (20%) were electrophoresed on 3% nussieve–1% agarose gels, in Tris–acetate buffer. Ethidium bromide staining of the RNA–PCR products is shown. M = DNA size marker. (+) = reactions carried out in the presence of reverse transcriptase. (−) = control reactions, without the enzyme, demonstrating the absence of contaminating DNA (one of two independent experiments).

the transgenic mice included $< 10^4$ molecules/μg based on the kinetic follow-up of this PCR amplification using in-vitro transcribed deleted BCHE RNA (G.Ehrlich *et al.*, unpublished observations). In contrast, the levels of the BCHE PCR product from somatic tail RNA were $> 10^5$ molecules/μg and indistinguishable in control and transgenic mice presenting no somatic amplifications (Figure 10). This evidence showed that the BCHE gene structure and its transcriptional ability in somatic tissues of the transgenic mice were normal, and focused the transcriptional damage to the testis tissue.

Discussion

We have observed in transgenic mice the occurrence of testicular gene amplification, the somatic amplification of the BCHE cDNA transgene which was limited to two generations and a concomitant variability in litter sizes which could reflect reduced fertility. The finding of somatic and testicular gene amplifications presented in this report demonstrates that in-vivo DNA amplifications may occur during germ cell development and are not limited to cancerous tissues and cultured cells. This extends recent observations of heritable CGG repeat expansion related with the X-linked mental retardation syndrome (Oberle *et al.*, 1991; Kremer *et al.*, 1991; Sutherland *et al.*, 1991) and CTG repeat expansion related to myotonic dystrophy (Brook *et al.*, 1992; Harley *et al.*, 1992).

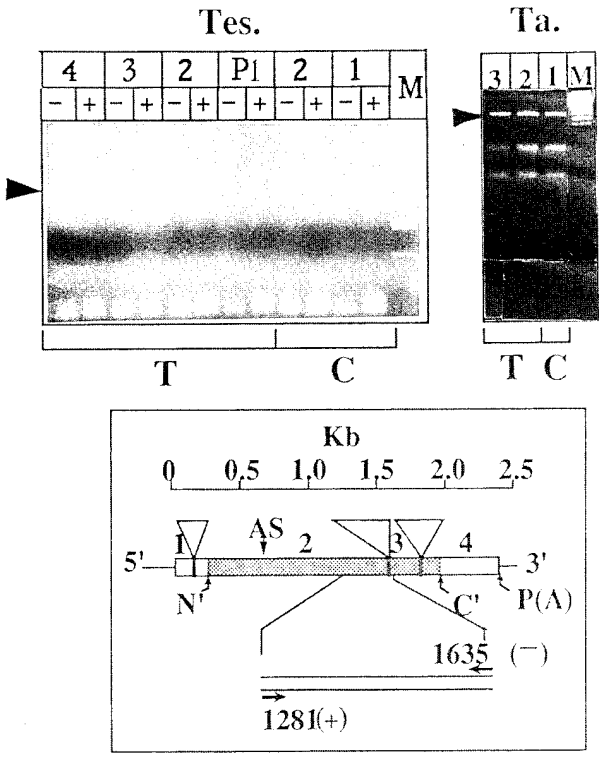

Fig. 10. Selective reduction in RNA−PCR-measured BCHE mRNA levels in testis of transgenic mice carrying amplified sequences. Experimental details were as in the legend to Figure 9, except that the following BCHE primers from exons 2 and 3 were employed: BCHE 1281(+), 5′-AGACTGGGTAGATGATCAGAG-ACCTGAAAACTACCG-3′ and 1635(−), 5′-GACAGGCCAGCTT GTGCTATTGTTCTGAGTCTCAT-3′, complementary to human BCHE cDNA (Prody *et al.*, 1987). Cycle numbers were 35 for tail BCHE mRNA (**right**), 42 for testis BCHE (**left**). See Arpagaus *et al.* (1990) for details on BCHE gene structure. One of two independent experiments.

The stable integration of the pSVL-CHE transgenic DNA into the mouse genome may be assumed to reflect a random event (Jaenisch, 1988). Yet, its amplification in two different pedigrees could have occurred by several mechanisms, independent of the insertion sites and thereby of sequences flanking the foreign DNA. SV-40 replication generally depends on the presence of the large T-antigen (Fiers *et al.*, 1978). Therefore, it is unlikely that this amplification was due to the SV-40 origin alone. Nevertheless, the unique transmission pattern of the amplified sequences is noteworthy, regardless of the initial cause for this phenomenon. The BCHE DNA sequence could have *cis*-activated randomly localized origins of replication in the host DNA adjacent to the transgene insertion sites. Alternatively, it could contain an intrinsic signal capable of directing its own amplification *in vivo*. If so, it could have replicated independently from the mouse genomic DNA during sperm development, when the host DNA is densely packed (Huret, 1986) and so less available for the incorporation of a foreign DNA into its chromatin structure. Interestingly, the 3q26 chromosomal site where the human BCHE gene resides (Gnatt *et al.*, 1990) carries an integration site for retroviruses (Soreq and Zakut, 1993). This site could hence be directly involved in the first amplification event of this gene in humans as well (Prody *et al.*, 1989).

Our findings raise the possibility that certain gene loci would be particularly vulnerable for germ cell gene amplification following viral infection. Initial changes in gene dosage by chromosome non-disjunction may trigger a general loss of accuracy in DNA replication (Holliday, 1989), and the co-amplification of several genes frequently occurs in cultured cells (Pauw *et al.*, 1986). Therefore, we consider that additional unexamined genes may also have undergone amplification in our model system. To the best of our knowledge, there are no previous examples of human DNA sequences undergoing amplification in transgenic mice; neither are there any indications that the amplification of a particular gene in transgenic mice may initiate a chain reaction resulting in the amplification of otherwise unlinked DNA sequences.

In view of our previous findings of ACHE, BCHE and c-*raf* co-amplifications in human cancers (Soreq and Zakut, 1990), one might suggest that the ACHE and BCHE genes, which are positioned on separate chromosomes in humans (Gnatt *et al.*, 1990; Ehrlich *et al.*, 1992), belong to an interrelated family of autosomal genes which co-amplify in various biosystems. Above a threshold copy number of one of these genes, the initial amplification event may have disrupted a natural equilibrium which under normal conditions prevents the uncontrolled amplification of multiple genes. This process could, for example, operate by the removal of an inhibitory protein element from the relevant DNA sites, explaining the similar extents of amplification among various dispersed endogenous amplifiable mouse genes, in FIII testis.

Our present observations imply that developing spermatozoa may be particularly vulnerable for cascade reactions of gene amplification, consistent with the variable litter sizes in the transgenic mice. The BCHE DNA amplifications observed in tail DNA from FII mice, and which presumably occurred in FI testis, do not appear to have imposed an impediment to their heritability. In contrast, the selective decrease in BCHE gene expression in the testis of the transgenic mice carrying multiple amplified genes apparently accompanied reduced fertilizing capacity of sperm cells carrying the amplifications. This could potentially be due to the amplification of additional genes, and particularly the endogenous ACHE gene. Interestingly, the ACHE and BCHE genes are co-regulated in multiple systems (Layer, 1991), perhaps indicating competition for common transcription factors, or direct or indirect feedback interactions involving the protein products. Either of these possibilities could implicate ACHE gene amplifications with reduced BCHE transcription.

The litter size variability observed in our transgenic mice reflects an apparent defect in fertility. Although qualitative evaluation failed to detect differences in sperm motility, we cannot exclude the possibility that minor yet effective changes in sperm motility did indeed occur. Parallel phenomena may hence occur in humans as well, particularly following viral infections and/or gene amplification events. This calls for further refinement of sperm motility measurements. Defects in human sperm motility, which are probably associated with cholinergic signalling (Rama Sastry *et al.*, 1981), constitute one of the primary factors in human male infertility (Chandley, 1988). The recently cloned promoter of the human ACHE gene (Ben Aziz-Aloya *et al.*,

1993) resembles promoters of other testicularly expressed genes in its contents of consensus motifs for binding transcription factors. If, indeed, CHE gene amplifications also occur in human sperm cells, they could induce defects in fertility and remain unnoticed. In contrast with mice, where the observed reduction in fertility was rather limited, parallel amplifications in humans may affect fertility more drastically because of the smaller size progeny. Other gene amplifications may similarly affect additional yet undefined vital processes in sperm development. The gonadal amplification phenomena observed in this study may potentially represent a case study of a general phenomenon which calls for clinical investigation in infertile humans.

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References

Arpagaus,M., Kott,M., Vatsis,K.P., Bartels,C.F., LaDu,B.N. and Lockridge,O. (1990) Structure of the gene for human butyrylcholinesterase. Evidence for a single copy. *Biochemistry*, **29**, 124–131.

Ben Aziz-Aloya,R., Seidman,S., Timberg,R., Sternfeld,M., Zakut,H. and Soreq,H. (1993) Expression of a human acetylcholinesterase promoter-reporter construct in developing neuromuscular junctions of *Xenopus* embryos. *Proc. Natl. Acad. Sci. USA*, **90**, 2471–2475.

Bishop,J.M. (1991) Molecular themes in oncogenesis. *Cell*, **64**, 235–248.

Brook,J.D., McCurrach,M.E., Harley,H.G., Buckler,A.J., Church,D., Aburatani,H., Hunter,K., Stanton,V.P., Thirion,J.P., Hudson,T., Sohn,R., Zemelman,B., Snell,R.G., Rundle,S.A., Crow,S., Davies,J., Shelbourne,P., Buxton,J., Jones,C., Juvonen,V., Johonson,K., Harper,P.S., Shaw,D.J. and Housman,D.E. (1992) Molecular basis of myotonic dystrophy: expansion of a trinucleotide (CTG) repeat at the 3′ end of a transcript encoding a protein kinase family member. *Cell*, **68**, 799–808.

Chandley,A.C. (1988) Meiosis in man. *Trends Genet.*, **4**, 79–84.

Cooper,D.N. and Schmidtke,J. (1987) Human gene cloning and disease analysis. *Lancet*, **i**, 273.

Delidakis,C., Swimmer,C. and Kafatos,F.C. (1989) Gene amplification: an example of genome rearrangement. *Curr. Opin. Cell Biol.*, **1**, 488–449.

Ehrlich,G., Viegas-Pequignot,E., Ginzberg,D., Sindel,L., Soreq,H. and Zakut,H. (1992) Mapping the human acetylcholinesterase gene to chromosome 7q22 by fluorescent *in situ* hybridization coupled with selective PCR amplification from a somatic hybrid cell panel and chromosome-sorted DNA libraries. *Genomics*, **13**, 1192–1197.

Fiers,W., Contreras,R., Haegeman,G., Rogiers,R., Van De Voora,A., Van Hauverswyn,H., Van Herreweghe,J., Volckaert,G. and Ysebaert,M. (1978) Complete nucleotide sequence of SV-40 DNA. *Nature*, **273**, 113–120.

Gnatt,A., Prody,C.A., Zamir,R., Lieman-Hurwitz,J., Zakut,H. and Soreq,H. (1990) Expression of alternatively terminated unusual CHEmRNA transcripts mapping to chromosome 3q26-ter in nervous system tumours. *Cancer Res.*, **50**, 1983–1987.

Harley,H.G., Brook,J.D., Rundle,S.A., Crow,S., Reardon,W., Buckler,A.J., Harper,P.S., Housman,D.E. and Shaw,D.J. (1992) Expansion of an unstable DNA region and phenotypic variation in myotonic dystrophy. *Nature*, **355**, 545–546.

Holliday,R. (1989) Chromosome error propagation and cancer. *Trends Genetics*, **5**, 42–45.

Huret,J.L. (1986) Nuclear chromatin decondensation of human sperm. *Arch. Androl.*, **16**, 97–109.

Ibanez,C.F., Petto-Huikko,M., Soder,O., Ritzen,E.M., Hersh,L.B., Hokfelt,T. and Persson,H. (1991) Expression of cholineacetyl transferase mRNA in spermatogenic cells results in an accumulation of the enzyme in the postacrosomal region of mature spermatozoa. *Proc. Natl. Acad. Sci. USA*, **88**, 3676–3680.

Jaenisch,R. (1988) Transgenic animals. *Science*, **240**, 1468–1474.

Kim,E., Waters,S.H., Hake,L.E. and Hech,N.B. (1989) Identification and developmental expression of a smooth-muscle actin in postmeiotic male germ cells in mice. *Mol. Cell Biol.*, **9**, 1875–1881.

Kremer,E.J., Pritchard,M., Lynch,M., Holman,Y.K., Baker,E., Warren,S.T., Schlessinger,D., Sutherland,G.R. and Richards,R.I. (1991) Mapping of DNA instability at the fragile X domain to a trinucleotide repeat sequence P(CCG)n. *Science*, **252**, 1711–1714.

Laat,S.A., LaLande,M. and Donlon,T. (1986) DNA-based detection of chromosome deletion and amplification: Diagnostic and mechanistic significance. *Cold Spring Harbor Symp. Quant. Biol.*, **LI**, 299–307.

Lapidot-Lifson,Y., Prody,C.A., Ginzberg,D., Maytes,D., Zakut,H. and Soreq,H. (1989) Co-amplification of human acetylcholinesterase and butyrylcholinesterase genes in blood cells: Correlation with various leukemias and abnormal megakaryocytopoiesis. *Proc. Natl. Acad. Sci. USA*, **6**, 4715–4719.

Lapidot-Lifson,Y., Patinkin,D., Prody,C.A., Ehrlich,G., Seidman,S., Ben-Aziz,R., Benseler,F., Eckstein,F., Zakut,H. and Soreq,H. (1992) Cloning and antisense oligodeoxynucleotide inhibition of a human homolog of cdc2 required in hematopoiesis. *Proc. Natl. Acad. Sci. USA*, **89**, 579–583.

Layer,P.G. (1991) Cholinesterases during development of the avian nervous system. *Cell. Mol. Neurobiol.*, **11**, 7–33.

Libermann,T.A., Nusbaum,H.R., Razon,N., Kirs,R., Lax,I., Soreq,H., Whitle,N., Waterfield,M.D. and Ullrich,A. (1985) Amplification, enhanced expression and possible rearrangement of EGF receptor gene in primary human tumours of glial origin. *Nature*, **313**, 144–147.

Makler,A. (1991) Sealed mini-chamber of variable depth for direct observation and extended evaluation of sperm motility under the influence of various genes. *Hum. Reprod.*, **6**, 1275–1278.

Mouches,C., Pasteur,N., Berge,J.B., Hyrien,O., Raymond,M., de Saint Vincent,B.R., de Silvestry,M. and Georghiou,G.P. (1986) Amplification of an esterase gene is responsible for insecticide resistance in a California Culex mosquito. *Science*, **233**, 778–780.

Nakqjima-Iijima,S., Hamada,H., Reddy,P. and Kakunaga,T. (1985) Molecular structure of the cytoplasmic ϒ-actin gene: Interspecies homology of sequences in the introns. *Proc. Natl. Acad. Sci. USA*, **82**, 6133–6137.

Oberle,I., Rousseau,F., Heitz,D., Kretz,C., Devys,D., Hanauer,A., Boue,J., Bertheas,M.F. and Mandel,J.L. (1991) Instability of a 550-base pair DNA segment and abnormal methylation in fragile X syndrome. *Science*, **252**, 1097–1102.

Pauw,P.G., Johnson,M.D., Moore,P., Morgan,MN., Finemon,R.M., Kalka,T. and Ash,J.F. (1986) Stable gene amplification and overexpression of sodium- and potassium-activated ATPase in Hela cells. *Mol. Cell Biol.*, **6**, 1164–1171.

Prody,C.A., Zevin-Zonkin,D., Gnatt,A., Goldberg,O. and Soreq,H. (1987) Isolation and characterization of full-length cDNA clones coding for cholinesterase from fetal human tissues. *Proc. Natl. Acad. Sci. USA*, **84**, 3555–3559.

Prody,C.A., Dreyfus,P., Zamir,R., Zakut,H. and Soreq,H. (1989) De novo amplification within a 'silent' human cholinesterase gene in a family subjected to prolonged exposure to organophosphorous insecticides. *Proc. Natl. Acad. Sci. USA*, **6**, 690–694.

Rama Sastry,B.V. and Sadavongvivad,C. (1979) Cholinergic systems

in non nervous tissues. *Pharmacol. Rev.*, **30**, 65–132.

Rama Sastry,B.V., Janson,V.E. and Chaturvedi,A.K. (1981) Inhibition of human sperm motility by inhibitors of cholineacetyl transferase. *J. Pharmacol. Exp. Ther.*, **216**, 378–384.

Richler,C., Soreq,H. and Wahrman,J. (1992) X inactivation in mammalian spermiogenesis is correlated with inactive X specific transcription. *Nature*, **2**, 192–195.

Schimke,R.T. (1990) The search for early genetic events in tumourigenesis: an amplification paradigm. *Cancer Cells*, **2**, 149–151.

Shani,M. (1985) Tissue specific expression of rat myosin light chain 3 gene in transgenic mice. *Nature*, **314**, 283–286.

Soreq,H. and Zakut,H. (1993) *Human Cholinesterases and Anti-Cholinesterases*. Academic Press, New York, 300 pp.

Soreq,H., Ben-Aziz,R., Prody,C.A., Seidman,S., Gnatt,A., Neville,L., Lieman-Hurwitz,J., Lev-Lehman,E., Ginzberg,D., Lapidot-Lifson,Y. and Zakut,H. (1990) Molecular cloning and construction of the coding region for human acetylcholinesterase reveals a G,C rich attenuating structure. *Proc. Natl. Acad. Sci. USA*, **87**, 9688–9692.

Stark,G.R., Dabatisse,M., Giulotto,E. and Wahl,G.M. (1989) Recent progress in understanding mechanisms of mammalian DNA amplification. *Cell*, **57**, 901–908.

Sutherland,R., Haan,E.A., Kremer,E., Lynch,M., Pritchard,M., Yu,S. and Richard,R.I. (1991) Hereditary unstable DNA: a new explanation for some old genetic questions? *Lancet*, **338**, 289–292.

Veyama,H., Hamada,H., Battula,N. and Kakunaga (1984) Structure of the human smooth muscle actin gene (aortic type) with a unique intron site. *Mol. Cell. Biol.*, **4**, 1073–1078.

Willison,K. and Ashworth,A. (1987) Mammalian spermatogenic gene expression. *Trends Genet.*, **3**, 351–355.

Zakut,H., Ehrlich,G., Ayalon,A., Prody,C.A., Malinger,G., Seidman,S., Ginzberg,D., Kehlenbach,R. and Soreq,H. (1990) Acetylcholinesterase and butyrylcholinesterase genes coamplify in primary ovarian carcinomas. *J. Clin. Invest.*, **6**, 900–908.

Zakut,H., Lapidot-Lifson,Y., Beeri,R., Ballin,A. and Soreq,H. (1992) In vivo gene amplification in non-cancerous cells: cholinesterase genes and oncogenes amplify in thrombocytopenia associated with lupus erythematosus. *Mutat. Res.*, **276**, 275–284.

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BRAIN RESEARCH

Research report

Cholinotoxic effects on acetylcholinesterase gene expression are associated with brain-region specific alterations in G,C-rich transcripts

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Abstract

To study the mechanisms underlying cholinotoxic brain damage, we examined ethylcholine aziridinium (AF64A) effects on cholinesterase genes. In vitro, AF64A hardly affected cholinesterase activities yet inhibited transcription of the G,C-rich AChE DNA encoding acetylcholinesterase (AChE) more than the A,T-rich butyrylcholinesterase (BChE) DNA. In vivo, intracerebroventricular injection of 2 nmol of AF64A decreased AChE mRNA in striatum and septum by 3- and 25-fold by day 7, with no change in BChE mRNA or AChE activity. In contrast, hippocampal AChE mRNA increased 10-fold by day 7 and BChE mRNA and AChE activity decreased 2-fold. By day 60 post-treatment, both AChE mRNA and AChE levels returned to normal in all regions except hippocampus, where AChE activity and BChE mRNA were decreased by 2-fold. Moreover, differential PCR displays revealed persistent induction, specific to the hippocampus of treated rats, of several unidentified G,C-rich transcripts, suggesting particular responsiveness of hippocampal G,C-rich genes to cholinotoxicity.

Keywords: Acetylcholinesterase; AF64A; Butyrylcholinesterase; Differential PCR display; Hippocampus; RT-PCR; Septum

1. Introduction

Cholinergic deficits have been associated with several neurodegenerative disorders such as Alzheimer and Huntington's diseases [33], suggesting that finely balanced cholinergic metabolism contributes to the maintenance of central nervous system circuits. Therefore, understanding the molecular and neurochemical changes underlying cholinergic signalling can assist in deciphering the causes for such diseases.

An animal model in which a selective cholinergic deficit has been induced is the ethylcholine aziridinium (AF64A) treated rat [6]. AF64A is structurally similar to choline, with the distinction that it possesses an ethyl moiety and an aziridinium ion. It is taken up by cholinergic neurons via the high affinity choline transport system [20] and causes a specific and long lasting reduction in the concentration and the activity of cholinergic pre-synaptic biochemical markers [21,28]. These markers include the neurotransmitter acetylcholine (ACh), the ACh hydrolysing enzyme acetylcholinesterase (AChE), the ACh synthesizing enzyme choline acetyltransferase (ChAT) [16], and the high affinity transport system for choline, which is the rate limiting step in ACh synthesis [22]. In addition, rats treated with AF64A also show behavioral cognitive deficits, suggesting that loss of synapses has occurred [2].

Referring to the analogy between AF64A and choline, and the presence of the highly reactive aziridinium ion which is susceptible to nucleophilic attack [12], it was suggested that AF64A serves as a potent inhibitor of enzymes that have affinity for choline, such as ChAT and AChE [29]. However, like other aziridinium compounds, AF64A was also found to react with DNA, to induce DNA damage and to cause premature termination of RNA transcription in vitro in a dose-dependent fashion. This probably occurs through direct interaction with the N-7 position in guanines in the DNA molecule [7]. Thus, the cholinotoxic effects of AF64A could be attributed to its in-

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hibitory action on cholinergic enzyme activities, to interference with the synthesis of such enzymes, to its DNA damaging activity on G,C-rich genes or to all of these actions together.

AChE and the closely related acetylcholine hydrolyzing enzyme, butyrylcholinesterase (BChE) are highly similar in their amino acid sequence (50% identity in humans) [31], and in their computer modelled three dimensional structure [10], yet vary in their substrate specificity and in their interaction with inhibitors [19,27]. In addition, the rat AChE gene is G,C-rich (59%) [14] like its human homolog [30], with high predicted sensitivity to guanine binding agents, whereas the BChE gene is A,T-rich (67%) [26] and may be expected to be less sensitive to such agents. This difference made the two cholinesterase genes appropriate models for the in vitro studies described in this report on the effectiveness of AF64A on cholinergic gene expression and/or enzymatic activities. In parallel, we further examined the effects of intracerebroventricular (i.c.v.) administration of AF64A on cholinergic enzyme activities and determined the in vivo effect of AF64A on cholinesterase mRNA levels by reverse transcription coupled with DNA amplification (RT-PCR) in different rat brain areas. To compare the pattern of expression of G,C-rich genes in brains from AF64A treated and control rats, differential PCR-displays [18] were prepared with an arbitrary G,C-rich primer [32]. Our findings suggest a consistent correlation between the cholinotoxic effects of AF64A on G,C-rich sequences in vitro and in vivo and suggest the use of this approach for identifying the target genes to cholinotoxic agents.

2. Materials and methods

2.1. Stereotactic surgery and i.c.v. AF64A infusion

Male Sprague–Dawley rats (Zivic Miller Laboratories, Allison Park, PA) weighing between 250 and 350 g were housed in groups of 2/cage, in a room that was maintained on a 12-h dark–light cycle. Animals had free access to water and food ad libitum. AF64A was prepared as previously described [6]. An aqueous solution of acetylethylcholine mustard HCl (1.0 nmol/l) was adjusted to pH 11.5 with NaOH and stirred at room temperature for 20 min, after which pH was brought to 7.3 with HCl, and the solution stirred at room temperature for another 60 min. This solution was prepared freshly prior to each experiment, and subsequent to these procedures the solution was kept on ice during the time required for surgery (up to 6 h).

Animals were anesthetized with chloral hydrate (350 mg/kg) and positioned in a Kopf small animal stereotactic frame. Two needles (26 gauge) were passed through parallel drilled holes in the skull and positioned bilaterally in the ventricles at the following stereotaxic coordinates from the bregma: posterior 0.8 mm, lateral ±1.5 mm, and ventral 3.6 mm. AF64A (2.0 nmol/1.5 μl) or an equal volume of vehicle, was infused bilaterally at a flow rate of 0.5 μl/min. The needles were left in place for 5 min after completion of the infusion; after which they were slowly pulled out.

2.2. Tissue preparation

At each predetermined time point post-AF64A administration the rat was decapitated, and its brain was removed and placed on an ice-cold surface. Specific brain regions were dissected out and frozen as soon as possible on dry-ice, then stored at −70°C until biochemical and molecular analyses could be carried out. Before the enzyme assay, tissues were thawed and homogenized in ice-cold sodium phosphate buffer (75 mM, pH 7.4, 1/20 w/v).

2.3. Cholinergic enzyme activity assays

ChAT activity assays were performed according to El-Tamer et al. [5]. Briefly, homogenate (10 μl) was added to 10 μl of buffer substrate mixture containing: sodium phosphate 75 mM (pH 7.4), NaCl 600 mM, $MgCl_2$ 40 mM, Eserine 2.0 mM, bovine serum albumin 0.05%, choline-iodide 10 mM, and [^{3}H]acetyl-coenzyme A 0.87 mM (18.6 mCi/mmol). After 30 min of incubation at 37°C, the tubes were placed on ice and 150 μl of sodium tetraphenylboron solution (75 mg/ml in 3-heptanone) was added to each tube in order to extract the newly synthesized radiolabeled ACh. Tubes were then vortexed, and after centrifugation 100 μl of the top organic layer were taken to measure the amount of [^{3}H]ACh extracted from the buffer, using liquid scintillation spectrometry. The amount of radioactivity extracted from buffer incubated in parallel, without tissue, was subtracted as blank.

2.4. Cholinesterase activities

Cholinesterase activities in brain tissues were measured according to the procedure adapted and described by Leventer et al. [15]. Alternatively, for the in vitro studies, AChE and BChE activities were measured spectrophotometrically as detailed elsewhere [24].

2.5. Statistical analysis

Statistical analysis of the data was performed by the one-way analysis of variance (ANOVA) and Ducan's Multiple-Range tests. Differences were considered significant if they had a P value of 0.05 or less.

2.6. In vitro transcription of AChE and BChE mRNAs in the presence of AF64A

Three transcription plasmids containing the cDNAs encoding for human cholinesterases were used: pSP64 BChE (Promega Corporation, Madison, WI) which contains the sp6 RNA polymerase binding site and hBChE coding sequence [24]; pGEM-ZF(+) (Promega Corporation, Madison, WI), which contains the T7 RNA polymerase binding site and the hAChE coding sequence [30,4] and a Bluescript SK(+) from Stratagene (La Jolla, CA) with T3 RNA polymerase binding site and the human BChE cDNA [24]. This latter plasmid was used to compare AF64A effects on transcription of a single cDNA primed by different RNA polymerases. Plasmid DNAs were incubated with different concentrations of AF64A for 60 min at 37°C. DNA was then purified by two ethanol precipitations. Transcription was performed using the transcription kit RPN #2006 from Amersham International (Buckinghamshire, England) on linearized transcription plasmids according to manufacturer's instructions and using the appropriate RNA polymerase. RNA molecules were radiolabeled by adding to the reaction mixture 10 μCi of [^{32}P]UTP (800 Ci/mmol) for each 5 μg of control or AF64A-treated DNA. Radiolabeled transcription products were denatured, electrophoresed on polyacrylamide gels (5%) containing 7 M urea and exposed to Kodak film autoradiography. Quantification of the transcription products

was performed by densitometric analysis of the film using the Soft Laser Scanning Densitometer Model SL-TRFF (Biomed Instruments, CA, USA) as detailed previously [13].

2.7. RT-PCR analysis of cholinesterase mRNA transcripts in different rat brain regions

Frozen brain regions were extracted by the RNAzol B (Cinna/Biotex Laboratories, Inc, Houston, TX) to yield total RNA according to manufacturer's instructions. Purified RNA samples were kept at −70°C. 100 ng of each RNA sample were subjected to reverse transcription using reverse transcriptase (Gibco BRL Life Technologies Inc. Gaithersburg, MD) followed by specific primer annealing and PCR conditions essentially as described elsewhere [17] with aliquots taken out every 4 cycles starting at cycle 26. PCR primers used in this experiment were 1522(+)/2003(−) and 271 (+)/457(−) for the human AChE and BChE genes, respectively, and 822(+)/996(−) for the rat β-actin, all as described elsewhere [17].

Quantification of the RT-PCR data was based on comparing the accumulation rate of PCR fragments deriving from the total RNA extraction to that resulting from in vitro transcribed specific RNA as detailed elsewhere [17].

2.8. Differential PCR display

The PCR display was essentially as described [32] except that 1 μg total RNA was used for each reaction and that the arbitrary primer used included 60% G,C residues: 5′-CCTCCGCGAGATCATCT-3′. Also, 25 mM of each dNTP was included and the annealing temperature for the high stringency cycles was 55°C. Exposure was for 1 day at room temperature.

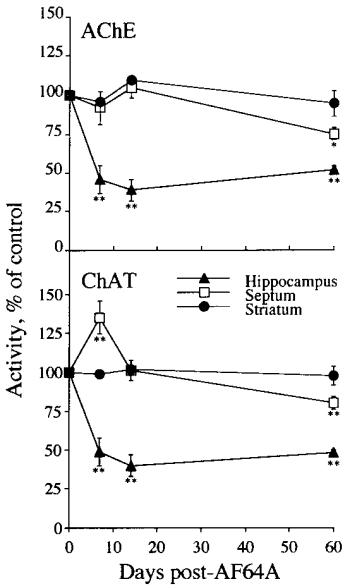

Fig. 1. In vivo effects of AF64A on cholinergic markers. Time-dependent effects of AF64A (2.0 nmol/side) on AChE and ChAT activities are presented for septum, hippocampus and striatum. Animals were sacrificed by decapitation at the indicated time after i.c.v. injection of AF64A. Enzymatic assays were performed in duplicate on tissue homogenates, all as detailed under Experimental Procedures. Data represent the average (mean ± S.E.M., $n = 3$ rats per group) of enzymatic activity expressed as percent of control. AChE activity in control group: mean ± S.E.M., 3476 ± 228, 9214 ± 281 and 20876 ± 474 nmol/mg/h in hippocampus, septum and striatum, respectively. ChAT activity in control group: mean ± S.E.M., 42.1 ± 3.5, 66.5 ± 5.2 and 209.7 ± 7.54 nmol/mg/h in hippocampus, septum and striatum, respectively. * $P < 0.05$; ** $P < 0.01$, compared to vehicle treated group.

3. Results

3.1. AF64A modulates cholinergic enzyme activities in vivo

AF64A effects on different cholinergic markers in vivo were first measured in a time-dependence study. To address the cholinergic deficits following i.c.v. administration of 2 nmol AF64A, we determined the enzymatic activities of AChE and ChAT in different brain regions. Septal AChE showed a slight but significant reduction (25%) which occurred late, at 60 days post-injection, while striatal AChE activity remained apparently unchanged until day 60. In contrast, AChE activity in hippocampus was reduced to 50–60% of control at day 7 through 60 post-AF64A administration (Fig. 1). The enzymatic activity of ChAT showed a different pattern: septal ChAT activity was significantly increased by 35% ($P < 0.01$) on day 7, returned to normal level by day 14 and decreased by 20% by day 60 post-AF64A treatment. In the striatum, ChAT activities remained normal after 0.5, 1.0 and 2 nmol/side AF64A at all of these time points (Fig. 1, and El-Tamer et al., unpublished data). However, hippocampal ChAT activity was significantly decreased to 40–50% of control level at day 7 through 60, corroborating previous observations [5]. The biochemical measurements thus revealed a particular long-term vulnerability to AF64A effects for cholinergic enzyme activities in the hippocampus.

3.2. Differential in vitro inhibition of cholinesterases by AF64A

In order to directly test the sensitivity of AChE and BChE to AF64A, we performed a kinetic spectrophotometric analysis of enzymatic activity following pre-incubation of AF64A in a concentration range of 1 to 1000 μM, with the tested enzymes. In this range of AF64A concentration our measurements fully reflected the interaction of the released thiocholine product with DTNB [24], and the color reduction under these experimental conditions was due to enzyme inhibition alone [9]. Following 45 min incubation of AChE and BChE with AF64A at room temperature, a dose-dependent reduction of the catalytic activity of these enzymes was observed (Fig. 2, Top). A significant inhibition of BChE was only detected at concentrations higher than 500 μM of the inhibitor and was limited to 32% inhibition at 1 mM AF64A. In contrast, AChE activity was sensi-

tive to concentrations of AF64A as low as 100 μM and 60% inhibition was caused in the presence of 1 mM AF64A (Fig. 2, Top). Similar results were obtained following incubation at a physiological temperature (37°C; results not shown). At the high concentration of AF64A used in vitro, one would expect susceptability to nucleophilic attack by cholinesterase activities. Moreover, at the physiologically effective average AF 64A concentration of 5–20 μM (based on administration of 2 nmol AF64A to approximately 160 ml CSF per ventricle, after Hebel and Stromberg [11]), neither of the enzymes should be inhibited. Therefore, we next focused our investigation on measurements at the mRNA level.

3.3. Pretreatment of cholinesterase cDNAs with AF64A causes differential damage to in vitro transcription

To compare the sensitivity of the AChE and BChE genes to AF64A, plasmid DNAs carrying each of these cholinesterase coding sequences were subjected to in vitro transcription following pre-incubation with 12.5–100 μM of AF64A. Significant reductions were observed in the yields of ^{32}P-labeled RNA transcripts from both AChE and BChE cDNA in a dose-dependent manner, however, to different extents. While yields of AChE mRNA transcripts were reduced to 76% of control following pre-incubation with as low as 12.5 μM of AF64A, 50 μM of this toxin were required to reduce BChE mRNA transcription to the same extent (Fig. 2, Bottom). The AChE gene therefore displayed differential sensitivity over that of the BChE gene toward AF64A toxicity in vitro, which predicted that physiologically effective concentrations of this cholinotoxin may modulate transcriptional activities of the corresponding genes in cholinergic and/or cholinoceptive cells.

3.4. AF64A administration modulates cholinesterase mRNA levels in vivo

To assess the in vivo levels of CHE mRNAs, total RNA extracts from septum, hippocampus and striatum from AF64A-treated and control rats were subjected to reverse transcription followed by kinetic followup of PCR amplification using primers specific to each of the cholinesterase genes (RT-PCR). As a control for the integrity of the examined mRNAs we followed the accumulation of β-actin mRNA. This analysis revealed that the amounts of septal and striatal AChE mRNA were reduced 25- and 3-fold at 7 days post-AF64A injection, respectively, and returned to 80 and 130% of control levels by day 60 (Fig. 3 and unshown data), while BChE mRNA levels remained unchanged (not shown). In contrast, hippocampal AChE mRNA was 10 times higher in treated rats at day 7 post-injection and returned to apparently normal levels (110%) at day 60. In this same region, BChE mRNA was reduced by 70% on day 7 and remained as low as 50% of control on day 60 (Fig. 3). Beta-actin mRNA products appeared in the RT-PCR tests 8 cycles before those of the cholinesterases, reflecting considerably higher levels than those of CHE mRNAs, and remained unchanged in septum and striatum after AF64A administration, demonstrating the specificity of the effects observed for CHE mRNAs. A 2-fold increase in β-actin mRNA was detected in the hippocampus, 7 days post AF64A administration (not shown), probably reflecting somewhat enhanced levels of general transcription in this cholinoceptive region. Although AChE activity was significantly reduced in the hippocampus, the RT-PCR analysis revealed a concomitant and pronounced increase in AChE mRNA, unique to this brain region.

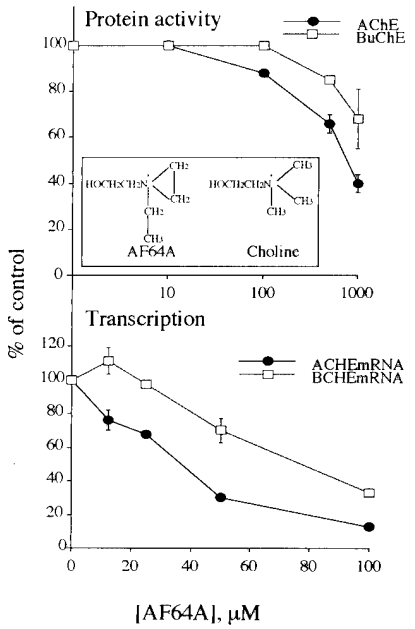

Fig. 2. In vitro effects of AF64A on mammalian CHE genes and their enzyme products. Top: direct AF64A induced inhibition of cholinesterase activities. Inhibition was measured following incubation with increasing concentrations of AF64A by determining remaining activities of AChE and BChE, all as detailed under methods. Inset: chemical structures of the cholinotoxin AF64A (a) and the native choline molecule (b), after Fisher et al. [6]. Bottom: differential sensitivity of the AChE and BChE genes for transcriptional damage induced by AF64A. Equal amounts of the noted plasmids incubated with the noted concentrations of AF64A were used for in vitro transcription in the presence of [^{32}P] nucleotides followed by agarose gel electrophoresis and autoradiogrephy of the ^{32}P-labeled reaction products. Note the relative resistance to AF64A of BChE – as compared with AChEcDNA. The same results were obtained for BChE mRNA transcribed from two transcription plasmids containing the human BChE coding sequences with two distinct RNA polymerases (not shown), demonstrating that the AF64A effects were due to the cDNA seqence and not to the type of RNA polymerase.

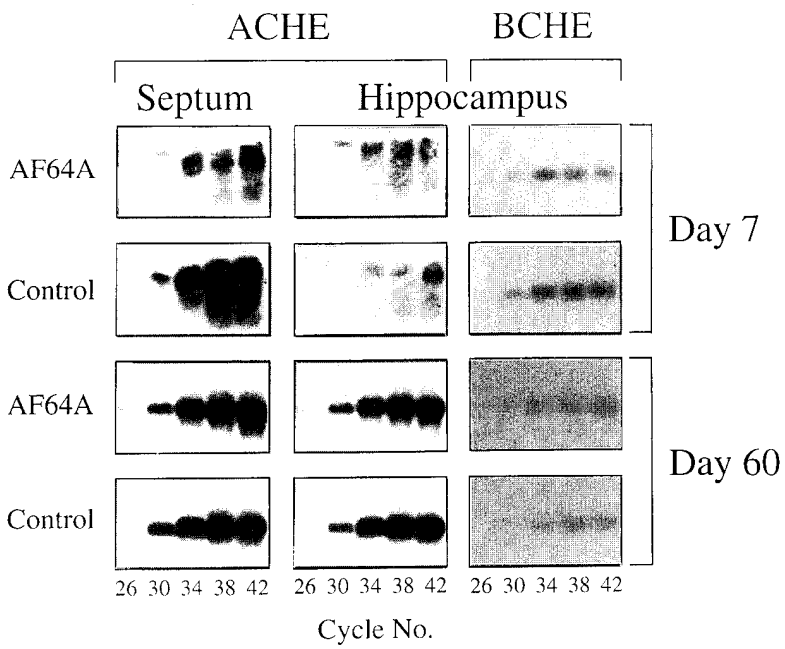

Fig. 3. In vivo modifications in cholinesterase mRNA levels following AF64A treatment. RNA was extracted from septum, hippocampus and striatum of 3 pooled animals 7 and 60 days post AF64A treatment and was subjected to RT-PCR procedure [17]. PCR products were detected as dark bands after hybridization followed by autoradiography.

3.5. AF64A induces region-specific alterations in the differential PCR display of G,C-rich transcripts

To examine whether the increased levels of AChE mRNA in 7 days treated hippocampus reflected a general change in transcription of G,C-rich genes, we employed the approach of differential PCR display [18,32]. To this end, we first examined whether this approach was sensitive enough to detect region-specific differences in G,C-rich transcripts. Amplification conditions were thus adapted so that differentially expressed transcripts were detected in the hippocampus, striatum and septum of control rats with an arbitrary G,C-rich primer. At least 50 conspicuous DNA products were detected after subjecting total RNA from each of these regions (pooled from three rats in each case) to first and second strand synthesis under low stringency annealing conditions, followed by PCR amplification (Fig. 4). A large part of the observed products was common to all regions, however, we also observed different PCR products which were dominant in specific regions (see Fig. 4 for striatum-specific products). Differential PCR displays from the same brain regions of individual animals confirmed that these bands were both dominant in specific regions and reproducible (data not shown).

The general pattern of displayed products did not change in the AF64A treated rats as compared to control rats (Fig. 4). While no AF64A-dependent

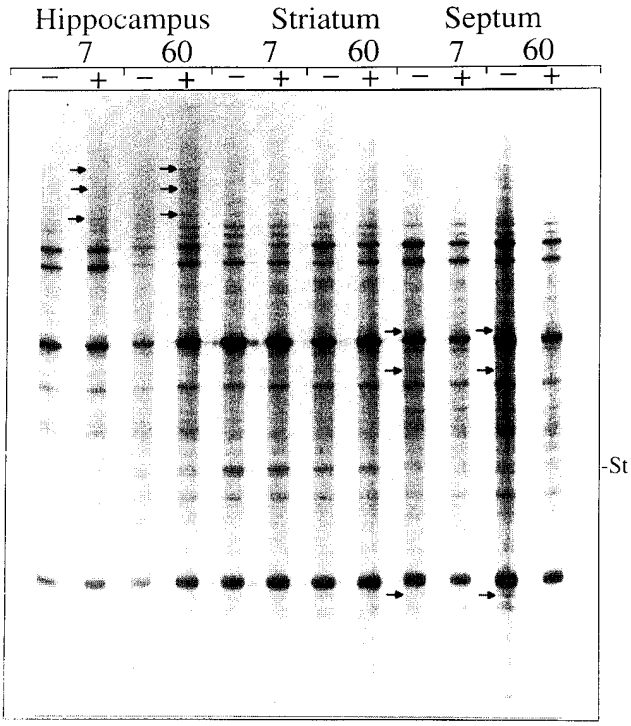

Fig. 4. PCR display. Differential PCR display of 1 μg total RNA extracted from hippocampus, striatum and septum pooled regions (from 3 animals/sample) of saline (–) and AF64A (+) injected rat brains, 7 and 60 days post treatment. Exposure was for 1 day at room temperature, for 10% of reaction mixture per lane. PCR fragments size was between 200 and 400 bp. Altogether most of the PCR products were common to the different brain regions; some were specific for either striatum (St) or hippocampus (not shown in this part of the gel). Arrows indicate PCR products whose intensity changed following AF64A injection.

changes could be observed in striatal mRNA, we were able to detect several quantitative changes in PCR products displayed from hippocampus and septum following AF64A administration. In the septum the levels of 3 transcripts were decreased on day 7 and remained low at day 60 (Fig. 4). At least 3 other PCR fragments appeared to be stronger in the treated as compared with non-treated hippocampus 60 days post AF64A. A less conspicuous but clear increase was also observed in these transcripts at day 7. Assuming that the PCR displays reflect the expression of over 30,000 distinct mRNA species in each brain region [23], this implies that the increase of AChE mRNA in the hippocampus was consistent with an increase in multiple G,C-rich transcripts which was particular to this cholinoceptive brain region.

4. Discussion

We found differential changes in activities of cholinergic marker proteins and in the levels of the corresponding mRNAs in the cholinoceptive hippocampus and the cholinergic septum and striatum of rats following i.c.v. administration of the cholinotoxin, AF64A. ChAT and AChE activities were conspicuously decreased in the hippocampus, reflecting damage to both processes and cell bodies. However, AChE mRNA and other unidentified G,C-rich mRNA transcripts were increased within hippocampal cell bodies, demonstrating a new and previously unforseen level of response to this cholinotoxic damage.

The in vivo experiments were complemented by a series of in vitro tests. In vitro inhibition of transcription of G,C-rich AChEcDNA was achieved at concentrations of AF64A that were 2 times lower than those required to inhibit the A,T-rich BChEcDNA, attributing at least part of the in vivo changes in AChE gene expression to its G,C-rich composition. In vitro studies further revealed that AChE activity was more sensitive to this alkylating agent than that of BChE, and that this inhibition required higher AF64A concentrations than those generally administered i.c.v. Moreover, access of such compounds to intracellular enzymes in the CNS parenchyma in the in vivo situation is very limited. Therefore, our in vitro tests suggest that to achieve direct inhibition of enzyme activities, the intracellular concentration of AF64A should be higher than those present in the CSF. The in vivo inhibition of AChE activity thus suggested that i.c.v.-administered AF64A actively accumulated in cholinergic synapses and entered into cholinergic and/or cholinoceptive cell bodies, where it could reach higher local concentrations. Our findings therefore support the theory of active uptake into cholinergic cell bodies via the choline transport system, which was previously suggested as a mechanism of action for AF64A [20,29]. Once in these cell bodies, AF64A is likely to interact with G,C-rich genes such as AChE and interfere with their transcription [7]. Moreover, the theory of active uptake further explains the apparent direct reduction of protein activities. Altogether, these findings imply that penetration of this alkylating agent into the brain can induce a multileveled cholinergic damage. This includes mechanisms of nucleophilic attack and blocking of guanines in the DNA at cholinergic cell bodies on the one hand, and interference with specific protein subsets at cell bodies and nerve terminals on the other hand. The damage caused by AF64A to DNA probably occurs through direct interaction with the N-7 position in guanines [7].

At the level of mRNA, the main damage induced in vivo by AF64A appeared to be region-specific and partially transient. That the transient decrease in AChE mRNA levels which was observed in cholinergic regions was probably limited to cholinergic cells was indicated from the observation that the average levels of the more ubiquitously expressed β-actin mRNA remained unchanged in spite of its equally high G,C-content (55%) [25]. At the protein level we could discriminate between the vulnerable hippocampus and the more resistant septum and striatum. Injection of 2 nmol AF64A/ventricle did not affect the activities of cholinergic markers measured in the striatum, which was previously found to be sensitive to higher doses of this cholinotoxin [28]. In contrast, the mRNA levels of both AChE, β-actin and other unidentified G,C-rich genes were significantly increased in the hippocampus by 7 days post AF64A administration. This indicated that the in vivo decrease in AChE activities in the hippocampus was secondary to the reduction in AChE mRNA in the septum, projecting to the hippocampus, and that this change caused a feedback increase of transcription of several genes, including AChE and β-actin, in this cholinoceptive and vulnerable area.

The hippocampus receives its main cholinergic innervation from medial-septal nuclei. Therefore, cholinoceptive cell bodies in this region should be sensitive to damage occurring to cholinergic neurons in the medial-septal nuclei. Cell bodies rich in AChE mRNA were indeed located by in situ hybridization to the CA1–3 regions and the dentate gyrus in rodent hippocampus (Lev-Lehman et al., unpublished data).

To evaluate the general transcriptional damage caused by AF64A, we adopted the recent approach of differential PCR display [18]. This strategy involves presentation of partial cDNA sequences, amplified from subsets of mRNAs by reverse transcription coupled to PCR using arbitrary primers [32]. Differentially expressed mRNAs were indeed observed in septum, striatum and hippocampus, and some of those particular to the hippocampus were modulated following

AF64A administration. The long term changes in gene expression detected by the PCR display suggest the use of this approach to clone and identify the modulated transcripts. That both the protein activities and the PCR display changes were long term can further indicate damage to the machinery of mRNA translation. This would reduce AChE activities even under conditions where AChE mRNA levels returned to normal, as was the case by day 60 post AF64A treatment.

The transcriptional changes in AF64A treated hippocampus are in line with the behavioral effects induced by this cholinotoxin. Moreover, prenatal administration of another alkylating agent, methylazoxymethanol (MAM) to pregnant rats at gestational day 14 or 15 resulted in a massive reduction in intrinsic cortical neurons and interneurons of hippocampus and striatum [3]. The enhanced transcription of β-actin and several other G,C-rich unidentified genes, in the AF64A treated hippocampus, therefore adds this cholinotoxin to the list of plasticity inducing agents, which calls for histological experiments to search for AF64A induced morphometric changes in the hippocampus.

Specific choline analogs can exert differential damage to cholinergic neurons through different mechanisms, such as selective block of receptors or interference with the functioning of genes encoding these protein products [1]. The fact that most of the cholinergic changes, on the mRNA level, were back to normal by 60 days post AF64A treatment supports the notion that cholinergic cells damaged by AF64A can largely recover and return to normal function. This reversibility can suggest the existence of intra-regional bypass machinery which responds to changes in the cholinergic balance by secondary regulation of gene transcription. Therefore, our present findings suggest the use of AF64A treated rats as a model for the cholinergic deficits occuring in Alzheimer's and Huntington's patients through characterization of the mRNA transcripts whose levels are modified in the septum and hippocampus after a single dose AF64A injection. The use of the sensitive PCR display method to decipher changes in gene expression can introduce a novel approach to the research of cholinergic deficits that will enable to pinpoint and characterize genes whose functioning is perturbed under conditions of cholinergic deficit.

Acknowledgements

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References

[1] Carmichael, W.W., The toxins of cyanobacteria, *Sci. Am.*, 270 (1994) 64–72.

[2] Chrobak, J.J., Hanin, I., Schmechel, D.E. and Walsh, T.J., AF64A-induced working memory impairment: behavioral, neurochemical and histological correlates, *Brain Res.*, 463 (1988) 107–117.

[3] Di-Luca, M., Merazzi, F., Cimino, M., Gispen, W.H., de Graan, P.N.E. and Cattabeni, E., Prenatally induced brain lesions, cognitive impairment and ptrotein-kinase C-dependent phosphorylation. In G. Racagni et al. (Eds.), *Biological Psychiatry*, Vol. 2, Elsevier, Amsterdam, 1991, pp. 188–191.

[4] Ehrlich, G., Ginzberg, D., Loewenstein, Y., Glick, D., Kerem, B., Ben-Ari, S., Zakut, H. and Soreq, H., Population diversity and distinct haplotype frequencies associated with AChE and BChE genes of Israeli Jews from Trans-Caucasian Georgia and from Europe, *Genomics*, in press.

[5] El-Tamer, A., Corey, J., Wulfert, E. and Hanin, I., Reversible cholinergic changes induced by AF64A in rat hippocampus and possible septal compensatory effect, *Neuropharmacology*, 31 (1992) 397–402.

[6] Fisher, A., Mantione, C.R., Abraham, D.J. and Hanin, I., Long term central cholinergic hypofunction induced in mice by ethylcholine aziridinium ion (AF64A) in vivo, *J. Pharm. Exp. Ther.*, 222 (1982) 140–145.

[7] Futscher, B.W., Pieper, O., Barnes, D.M., Hanin, I. and Erickson, L.C., DNA damaging and transcription-terminating lesions induced by AF64A in vitro, *J. Neurochem.*, 58 (1992) 1504–1509.

[8] Gall, C., Murray, K. and Isackson, P.J., Kainic acid-induced seizures stimulate increased expression of nerve growth factor mRNA in rat hippocampus, *Mol. Brain Res.*, 9 (1991) 113–123.

[9] Hanin, I., Yaron, A., Ginzberg, D. and Soreq, H., AF64A attenuates human acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) gene transcription in vitro. In I. Hanin, A. Fisher and M. Yoshida, (Eds.), *Alzheimer's and Parkinson's Diseases: Recent Advances*, Plenum, NY, 1994, in press.

[10] Harel, M., Sussman, J.L., Krejci, E., Bon, S., Chanal, P., Massoulie, J. and Silman I., Conversion of acetylcholinesterase to butyrylcholinesterase: modeling and mutagenesis, *Proc. Natl. Acad. Sci. USA*, 89 (1992) 10827–10831.

[11] Hebel, R. and Stromberg, M.W. (Eds.), *Anatomy of the Laboratory Rat*, The Williams and Wilkins Company, Baltimore, Waverly Press, 1976, pp. 124–125.

[12] Hortnagl, H., Potter, P.E., Happe, K., Goldstein, S., Leventer, S., Wulfert, E. and Hanin, I., Role of aziridinium moiety in the in vivo cholinotoxicity of ethylcholine aziridinium ion (AF64A), *J. Neurosci. Methods*, 23 (1988) 101–105.

[13] Lapidot-Lifson, Y., Prody, C.A., Ginzberg, D., Meytes, D., Zakut, H. and Soreq, H., Coamplification of human acetylcholinesterase and butyrylcholinesterase genes in blood cells: Correlation with various leukemias and abnormal megakaryocytopoiesis, *Proc. Natl. Acad. Sci. USA*, 86 (1989) 4715–4719.

[14] Legay, C., Bon, S., Vernier, P., Coussens, F. and Massoulie, J., Cloning and expression of a rat acetylcholinesterase subunit: generation of multiple molecular forms and complementarity with a *Torpedo* collagenic subunit, *J. Neurochem.*, 60 (1993) 337–346.

[15] Leventer, S.M., McKeag, D., Clancy, M., Wulfert, E. and Hanin, I., Intracerebroventricular administration of ethylcholine mustard aziridinium ion (AF64A) reduces release of acetylcholine from hippocampal slices, *Neuropharmacology*, 24 (1985) 453–459.

[16] Leventer, S.M., Wulfert, E. and Hanin, I., Time course of ethylcholine aziridinium ion (AF64A)-induced cholinotoxicity in vivo, *Neuropharmacology*, 26 (1987) 361–365.

[17] Lev-Lehman, E., Hornreich, G., Ginzberg, D., Gnatt, A., Meshorer, A., Eckstein, F., Soreq, H. and Zakut, H., Antisense inhibition of acetylcholinesterase gene expression causes transient hematopoietic alterations in vivo, *Gene Therapy*, 1 (1994) 127–135.

[18] Liang, P. and Pardee, A.B., Differential display of eukaryotic messenger RNA by means of polymerase chain reaction, *Science*, 257 (1992) 967–971.

[19] Loewenstein, Y., Gnatt, A., Neville, L.F. and Soreq, H., Chimeric human cholinesterase: identification of reaction sites responsible for recognition of acetyl- or butyrylcholinesterase – specific ligands, *J. Mol. Biol.*, 234 (1993) 289–296.

[20] Mantione, C.R., Fisher, A. and Hanin, I., The AF64A-treated mouse: possible model for cholinergic hypofunction, *Science*, 213 (1981) 579–580.

[21] Mantione, C.R., Zigmond, M.J., Fisher, A. and Hanin, I., Selective presynaptic cholinergic neurotoxicity following intrahippocampal AF64A injection in rats, *J. Neurochem.*, 41 (1983) 251–255.

[22] Murrin, L.C., High-affinity transport of choline in neural tissue, *Pharmacology*, 21 (1980) 132–140.

[23] Nedivi, E., Hevroni, D., Naot, D., Israeli, D. and Citri, Y., Numerous candidate plasticity-related genes revealed by differential cDNA cloning, *Nature*, 363 (1993) 718–722.

[24] Neville, L.F., Gnatt, A., Loewenstein, Y., Seidman, S., Ehrlich, G. and Soreq, H., Intramolecular relationships in cholinesterases revealed by oocyte expression of site-directed and natural variants of human BChE, *EMBO. J.*, 11 (1992) 1641–1649.

[25] Nudel, U., Zakut, R., Shani, M., Neuman, S., Levi, Z. and Yaffe, D., The nucleotide sequence of the rat cytoplasmic beta-actin gene, *Nucl. Acids Res.*, 11 (1983) 1759–1771.

[26] Prody, C.A., Zevin-Sonkin, D., Gnatt, A., Goldberg, O. and Soreq, H., Isolation and characterization of full-length cDNA clones for cholinesterase from fetal human tissues, *Proc. Natl. Acad. Sci. USA*, 84 (1987) 3555–3559.

[27] Quinn, D., Acetylcholinesterase: enzyme structure, reaction dynamics and virtual transion states, *Chem. Rev.*, 87 (1987) 955–979.

[28] Sandberg, K., Hanin, I., Fisher, A. and Coyle, J.T., Selective cholinergic neurotoxin: AF64A's effects in rat striatum, *Brain Res.*, 293 (1984) 49–55.

[29] Sandberg, K., Schnaar, R.L., Hanin, I. and Coyle, J.T., AF64A: an active site directed irreversible inhibitor of choline acetyltransferase, *J. Neurochem.*, 44 (1985) 439–445.

[30] Soreq, H., Ben-Aziz, R., Prody, C., Seidman, S., Gnatt, A., Neville, L., Lieman-Hurwitz, J., Lev-Lehman, E., Ginzberg, D., Lapidot-Lifson, Y. and Zakut, H., Molecular cloning and construction of the coding region for human acetylcholinesterase reveals a G + C rich attenuating structure, *Proc. Natl. Acad. Sci. USA*, 87 (1990) 9688–9692.

[31] Soreq, H. and Zakut, H., *Human Cholinesterases and Anticholinesterases*, Academic Press, NY, 1993. pp. 1–314.

[32] Welsh, J., Chada, K., Dalal, S., Cheng, R., Ralph, D. and McClelland, M., Arbitrary primed PCR fingerprints of RNA, *Nuc. Acid Res.*, 20 (1992) 4965–4970.

[33] Wurtman, R.J., Choline metabolism as a basis for the selective vulnerability of cholinergic neurons, *Trends Neurosci.*, 15 (1992) 117–122.

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MOLECULAR BRAIN RESEARCH

Successive organophosphate inhibition and oxime reactivation reveals distinct responses of recombinant human cholinesterase variants

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Abstract

To explore the molecular basis of the biochemical differences among acetylcholinesterase (AChE), butyrylcholinesterase (BuChE) and their alternative splicing and allelic variants, we investigated the acylation phase of cholinesterase catalysis, using phosphorylation as an analogous reaction. Rate constants for organophosphate (DFP) inactivation, as well as for oxime (PAM)-promoted reactivation, were calculated for antibody-immobilized human cholinesterases produced in *Xenopus* oocytes from natural and site-directed variants of the corresponding DNA constructs. BuChE displayed inactivation and reactivation rates 200- and 25-fold higher than either product of 3'-variable AChE DNAs, consistent with a putative in vivo function for BuChE as a detoxifier that protects AChE from inactivation. Chimeric substitution of active site gorge-lining residues in BuChE with the more anionic and aromatic residues of AChE, reduced inactivation 60-fold but reactivation only 4-fold, and the rate-limiting step of its catalysis appeared to be deacylation. In contrast, a positive charge at the acyl-binding site of BuChE decreased inactivation 8-fold and reactivation 30-fold. Finally, substitution of Asp70 by glycine, as in the natural 'atypical' BuChE variant, did not change the inactivation rate yet reduced reactivation 4-fold. Thus, a combination of electrostatic active site charges with aromatic residue differences at the gorge lining can explain the biochemical distinction between AChE and BuChE. Also, gorge-lining residues, including Asp70, appear to affect the deacylation step of catalysis by BuChE. Individuals carrying the 'atypical' BuChE allele may hence be unresponsive to oxime reactivation therapy following organophosphate poisoning.

Keywords: Organophosphate; Oxime reaction; Acetylcholinesterase; Butyrylcholinesterase; Diisopropylfluorophosphonate; Pyridine-2-aldoxime methiodide

1. Introduction

In spite of access to the 3-dimensional structure [6,29] and ample experimental [3–6,12–19,31] and theoretical [3,15,20,22–28] work, the biological implications of the complex catalytic process of cholinesterases (ChEs) are far from being fully understood. There are two human ChE genes, ACHE and BCHE, both of which have been cloned and expressed (reviewed by Soreq and Zakut [28]). These encode the homologous acetylcholinesterase (AChE, EC 3.1.1.7) and butyryl-

Abbreviations: ABS, acyl-binding site; AChE, acetylcholinesterase; BChE, butyrylcholinesterase; BTCh, butyrylthiocholine; CBS, choline-binding site; ChE, cholinesterase; DFP, diisopropylfluorophosphonate; DIP, diisopropylphosphoryl; ELISA, enzyme-linked immunosorbant assay; E5, the product of the mRNA encoding exons 2, 3, 4, intron 4 and exon 5; E6, the product of the mRNA encoding exons 2, 3, 4, and 6; OP, organophosphorus agent; PAM, pyridine-2-aldoxime methiodide; tAChE, Torpedo AChE.

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cholinesterase (BuChE, EC 3.1.1.8) proteins. The active site in both enzymes is positioned at the bottom of a deep gorge lined with hydrophobic residues [6,29]. However, although both these enzymes hydrolyze their substrates at exceptionally fast rates [20,22], they display clear differences in substrate specificity and inhibitor interactions [6,27,31]. In the case of BuChE, the relatively less aromatic gorge is lined with fewer aryl groups and more alkyl residues [6,29]. The catalytic triad, its adjacent choline- and acyl-binding sites and the surrounding residues determine the characteristic specificities of these enzymes to substrates and inhibitors [6,18,20,31]. An appreciation of the molecular basis of the differences between AChE and BuChE, therefore, requires a consideration of the contributions of specific residues both in the gorge lining and in the active site regions of the two enzymes.

ChE catalysis is a multi-step process, which includes attraction of the substrate into the deep gorge, formation of an acyl-enzyme intermediate by displacement of the choline alkoxy group by the hydroxyl oxygen of the enzyme's active site serine, exchange of water for the choline molecule, and hydrolysis of the acyl-enzyme (Figs. 1 and 2A). Detailed dissection of catalysis by any natural or recombinant ChE variant [18,19,28,31] should attempt to discriminate among effects on one or another of these stages of catalysis. As the slowest of these stages has a half-time in the order of 100 μs [3,20,22], we have recruited the inactivation of ChEs by an organophosphorus agent (OP) [30] as an analogous reaction in order to study in isolation the first of these two stages of catalysis (Fig. 1A). OP phosphorylation of the active site serine [19] is just as specific for S^{198} (sequence numbers for human BuChE [28]; S^{198} of human BuChE is homologous to S^{200} of *Torpedo* AChE [23]) as is the acylation stage of catalysis. Furthermore, it also has a pH/rate profile shaped by dependence on a conjugate base of pK_a near 7.2 and a conjugate acid of pK_a near 9.3 [35]. Thus, the same features of AChE that promote its acylation should facilitate its phosphorylation.

Cleavage of the phosphoryl-ChE bond is extremely slow, making OPs hemi-substrate inhibitors, but the rate of dephosphorylation can be enhanced by rationally designed nucleophiles, such as pyridine-2-aldoxime methiodide (PAM, Fig. 1B) [35]. PAM is a rigid zwitterionic molecule that juxtaposes its nucleophilic group precisely against the phosphoryl bond, which it displaces. That PAM acts from the choline binding site is indicated by choline being a competitive inhibitor [22], and by natural substrates competing with PAM in the reactivation reaction [12]. Moreover, the order of effectiveness of non-assisted hydrolysis of the spectrum of dialkylphosphoryl-ChEs formed by OP agents (dimethyl > diethyl > diisopropyl) is maintained in the PAM-assisted reactivations [30]. In spite of the obvious steric and electronic differences between PAM and a water molecule, PAM-promoted reactivation thus shares mechanistic characteristics with the deacylation step of catalysis.

Because BuChE is prevalent in serum, it can react easily with anti-ChEs before they have a chance to

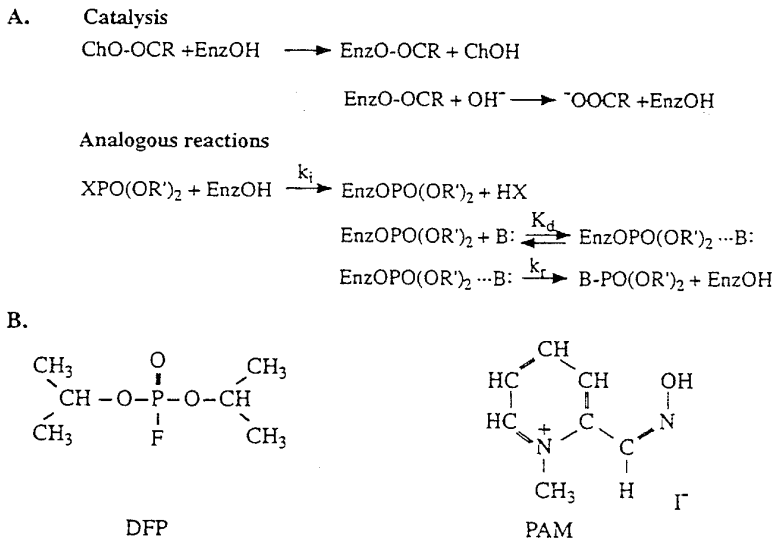
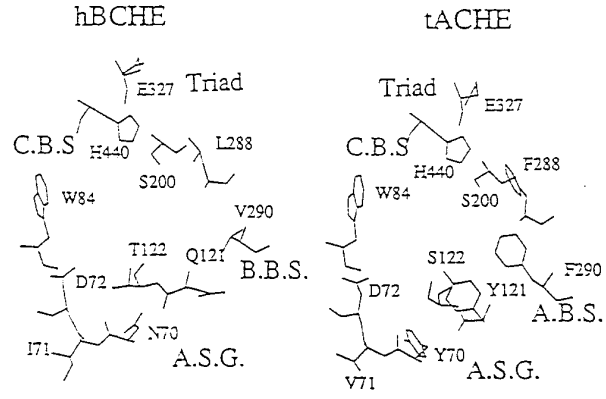


Fig. 1. The experimental paradigm. A: catalysis and analogous reactions. In the catalytic cycle the enzyme's active site serine (Enz-OH) displaces the choline moiety of the substrate, forming an acyl-enzyme (covalent) intermediate. The OP agents, being hemi-substrates, act analogously: serine displaces the leaving group (X), forming a dialkylphosphoryl-enzyme [30]. Catalysis continues with the hydrolysis of the acyl enzyme, whereas hydrolysis of the phosphoryl-ChE bond is extremely slow. The reactivation rate, however, can be enhanced by nucleophiles (B) such as choline and PAM, which actively displace the phosphoryl group. B: structure of DFP and PAM.



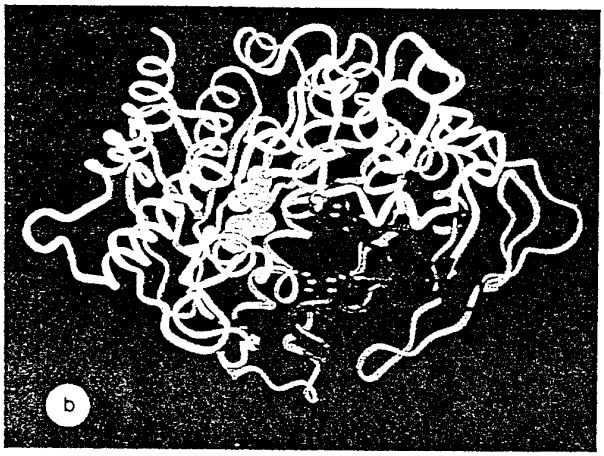


Fig. 2. The analyzed regions. A: active site environment in AChE and BuChE. Active site residues in *Torpedo* AChE (right, tACHE) and human BuChE (left, hBCHE) are shown following the existing structure and numbering of residues in *Torpedo* AChE [29] and the computer model of human BuChE6 [5]. Note differences in the acyl-binding site (ABS) and in the number of aromatic residues, and the similarity in the choline-binding site (CBS) and the catalytic triad (BuChE residues S^{198}, H^{438} and E^{327}). L^{288} in *Torpedo* is equivalent to L^{286} in human BuChE. Numbering of AChE residues is as for the *Torpedo* enzyme [28,29]. B: positioning of the replaced regions: The ribbon model of human BuChE was drawn after Harel et al. [6] and as detailed elsewhere [13,27]. The active site gorge lies in the plane of the figure, with its opening at the bottom. The substituted chimeric peptide [13] is shown in ~~turquoise~~ dark gray site-directed (~~red~~) and natural (~~blue~~) mutations were introduced in positions indicated as space-filling models, counterclockwise: S^{425} (~~blue~~, bottom left), D^{70} (~~blue~~, center), Y^{114} (~~blue~~, top), L^{286} (~~red~~, upper) and F^{329} (~~red~~, lower). Natural point mutations included D^{70} on the rim of the gorge, Y^{114} in the chimeric region, and S^{425}.

inactivate AChE at brain synapses and neuromuscular junctions. However, in addition to the differences between AChE and BuChE, several allelic variants of BuChE differ in their interactions with natural (e.g. glyco-alkaloids of the *Solanaceae*) and man-made anti-ChEs (e.g. OP and carbamate pesticides) [28]. Succinylcholine, which is used as a muscle relaxant prior to anesthesia, is slowly hydrolyzed by normal BuChE, but the fairly common $D^{70}G$ ('atypical') variant of BuChE does not hydrolyze it [2,16,17], resulting in post-operative apnea in individuals homozygous for this variant. This raises the question whether individuals with this and other variant BuChEs, when exposed to anti-ChEs, such as pesticides, pharmaceuticals, etc., will react differently than those with the common BuChE.

To analyze catalysis on a micro scale and to avoid interference with rate measurements by inactivating or reactivating reagents, we immobilized recombinant *Xenopus* oocyte-produced variant ChEs on selective monoclonal antibodies in multi-well plates and subjected the bound enzymes to successive OP inactivation and oxime-promoted reactivation. By this approach we measured major changes in rates of the reaction with diisopropylfluorophosphonate (DFP) and PAM (Fig. 1B) for a large series of human ChE variants differing within the gorge lining, the acyl-binding site or the C-terminus, always by comparison to the wild-type enzyme forms. Thus, we were able to implicate several regions of ChEs in particular stages of catalysis based on changes in rates of inactivation and reactivation, and predict anomalous responses to ChE inhibitors for individuals carrying the 'atypical' BuChE variant.

2. Materials and methods

2.1. Chemicals

Monoclonal mouse anti-human AChE and BuChE antibodies were purified as detailed [11]. Polyclonal rabbit anti-human BuChE was a product of Dako (Glostrup, Denmark). The horseradish peroxidase (HRP) conjugate of goat anti-rabbit immunoglobulin was from Jackson Laboratories (Bar Harbor, ME); DFP was a product of Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were the highest quality available from Sigma Chemical Co. (St. Louis, MO).

2.2. Mutants of BuChE

Production of natural and site-directed variants of cholinesterases and their expression in *Xenopus* oocytes has been described [4,8,13,17,18]. The enzyme samples represent products of 3 independent microinjections of mRNA for BuChE and for each of its mutants, 2 microinjections of AChE (E6) DNA [24]. 1 of AChE (E5) DNA [8], and 3 microinjections of mRNA of the BuChE/AChE chimera [13].

2.3. Immobilization of recombinant enzymes

Mouse anti-human serum BuChE or monoclonal anti-human AChE antibodies [11] were adsorbed to

These changes were made necessary when, upon the editor's advice, we abandoned the color illustration. We hope the half-tone illustration will be of better quality than this photocopy.

microtiter plates (Nunc, Roskilde, Denmark) at 0.5 μg/ml in 0.1 M carbonate buffer, pH 9.6, for at least 4 h at room temperature. Plates were then washed 3 times in PBS-T buffer (144 mM NaCl, 20 mM Na phosphate, pH 7.4, 0.05% Tween-20). Free binding sites on the well surface of the microtiter plate were blocked with PBS-T for 1 h at 37° C. Microinjected oocyte homogenates containing the enzyme were then added at a dilution of 1:20 in PBS-T or at a concentration of 100 mIU/ml in PBS-T for at least 3 h at room temperature with agitation. Plates were washed 3 times with PBS-T before use.

2.4. Inactivation of immobilized enzymes

Inactivation, reactivation and assay were all performed at pH 7.4. Immobilized enzymes were exposed to DFP in PBS-T buffer for varying times. Activity was always measured at 30 mM butyrylthiocholine, which was at least 10 times the K_m value for BuChE and the natural mutants, and 1.5- to 5-times that of the site-directed mutants [4]. Activity of AChE (E6 and E5) was measured at 2 mM acetylthiocholine.

2.5. Reactivation of inactivated immobilized enzymes

Determination of rate constants for reactivation is complicated by ageing, the progressive refractoriness of OP-inhibited enzyme to reactivation [19,30]. Once hydrolysis of one of the two alkyl groups occurs, the product, monoalkyl phosphoryl-ChE, is resistant to reactivation. The aging process proceeds concurrently with reactivation. In order to minimize the extent of ageing, inactivations were performed at sufficiently high DFP concentrations to bring residual activities to below 2% of the uninhibited level within 10 min, and reactivations were begun as soon as possible, usually within 5 min. The wells containing diisopropylphosphoryl (DIP)-ChE were exposed to 1 mM PAM in PBS-T, 22° C for various times, then washed several times with PBS-T and assayed for enzyme activity.

2.6. Assay of immobilized cholinesterases

Spectrophotometric assessment of hydrolysis rate in 96-well microtiter plates was performed as described [4,17,18,24]. To each well were added 200 μl 30 mM butyrylthiocholine (or, in the case of AChE, 2 mM acetylthiocholine) in 0.5 mM 3,3′-dithiobis(6-nitrobenzoic acid) (DTNB), 100 mM Na phosphate, pH 7.4. Absorbance at 405 nm was automatically recorded on a Molecular Devices microtiter plate reader (Menlo Park, CA).

2.7. Calculation of rate constants

Rate constants for inactivation (k_i) were calculated by linear regression analysis of $\ln(A_t)$ vs. t, where t is the time of exposure to DFP, and A_t is the remaining activity at time t. The pseudo-first order rate constant

Table 1
Kinetic rate constants for DFP-inactivation, PAM-reactivation and catalysis of cholinesterases [a]

Variant	$k_i \times 10^{-4}$ (M^{-1} min^{-1})	$k'_r \times 10^3$ (min^{-1})	$k_{cat} 10^{-3}$ (min^{-1})
BuChE	1220 ± 4 (3)	150 ± 30 (11)	96 ± 22 (6)
Chimera	19 ± 8 (2)	40 ± 18 (6)	36 ± 14 (5)
AChE (E6)	7 ± 1 (4)	6 ± 2 (3)	7.5 [c]
AChE (E5)	5 (1)	8 ± 3 (2)	
$L^{286}D$	188 ± 24 (3)	120 ± 30 (3)	38 ± 15 (5)
$L^{286}Q$	166 ± 40 (3)	120 ± 20 (4)	~~12 ± 4 (5)~~ 42 ± 24 (5)
$L^{286}R$	268 ± 164 (3)	6 ± 3 (3)	13 ± 7 (5)
$L^{286}K$	138 ± 4 (3)	4 ± 1 (3)	13 ± 5 (4)
$F^{329}R$		43 (1)	
$F^{329}Q$	1398 ± 532 (3)	44 ± 15 (5)	
$F^{329}C$	552 ± 408 (2)	14 ± 2 (4)	
$F^{329}D$	442 ± 190 (3)	8 ± 1 (4)	
$S^{425}P$ [b]	1054 ± 408 (3)	134 ± 8 (4)	
$D^{70}G$ [b]	1008 ± 418 (3)	32 ± 4 (4)	
$D^{70}G + Y^{114}H$ [b]	2112 ± 1074 (3)	12 ± 5 (3)	
$D^{70}G + S^{425}P$ [b]	260 ± 12 (2)	11 (1)	
$D^{70}G + Y^{114}H + S^{425}P$ [b]	1598 ± 294 (3)	4 ± 1 (3)	

[a] Second order rate constants for inhibition (k_i) were calculated for each of the noted variants of human ChEs from rates observed between 1 nM and 1 μM DFP, as in Fig. 3A. Pseudo-first order rate constants for reactivation (k'_r) were calculated from rates observed at 1 mM PAM, as in Fig. 3B. k_{cat} values were calculated from the rates of reaction with 30 mM butyrylthiocholine and the quantity of enzyme evaluated by ELISA assay of the enzyme, using known amounts of human serum BuChE to construct a standard curve. Numbers of experiments, in parentheses, and standard deviations are shown.

[b] Natural variant of BuChE.

[c] Taken from Ordentlich et al. [19].

for reactivation, k_r, was calculated by linear regression analysis of $\ln(A_\infty - A_i)/(A_\infty - A_t)$ vs. t, where t is the time of exposure to PAM, and A_∞ is the potential activity, A_t, the activity at time t, and A_i, the residual activity of the inhibited enzyme.

2.8. Quantification of immobilized cholinesterase

A rabbit anti-human BuChE polyclonal antibody was added to the immobilized ChE at 1:4,000 dilution in PBS-T for 1 h at 37° C. After washing with PBS-T, a goat anti-rabbit antibody conjugated to HRP was added at 1:10,000 dilution in PBS-T. HRP activity was assayed by using *o*-phenylenediamine dihydrochloride at 1 mg/ml in phosphate/citrate buffer, pH 9.6, and Na perborate as substrate. Purified human BuChE was used as a standard. The absorbance at 450 nm was recorded on a Molecular Devices microtiter plate reader.

3. Results

3.1. Spatiotemporal dissociation of catalytic steps

Since residues in the gorge lining and the acyl-binding site of human BuChE differ from those in AChE (Fig. 2A), we first focussed on the involvements of these regions in the catalytic process. To this end, we collected rate constants for each of the two recombinant human enzymes as produced in microinjected *Xenopus* oocytes from the corresponding cloned in vitro transcribed mRNA [18] or cDNA [24]. To further dissect the differences between human AChE and BuChE, we also examined a chimera in which the gorge rim, the gorge lining, the conserved oxyanion hole and the choline-binding site of BuChE were substituted with the homologous peptide of AChE [13]. The major difference in the chimeric enzyme was that its gorge lining was more aromatic than BuChE. To

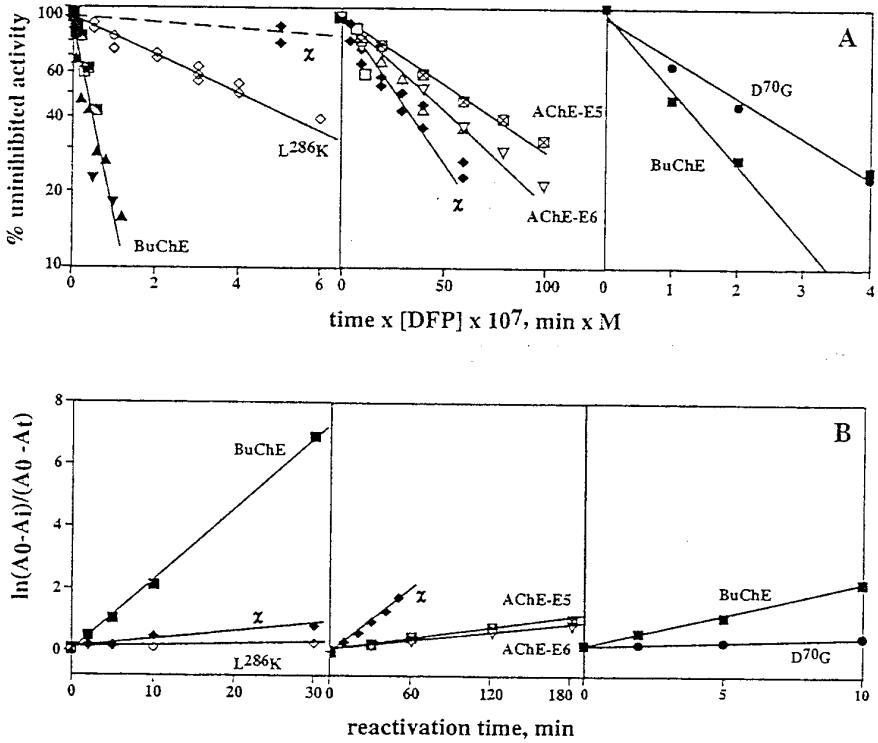


Fig. 3. Inactivation of ChEs and reactivation by PAM. A: data are presented as percent original activity vs. duration of exposure to DFP times the DFP concentration (k_i [DFP]). In the left panel, a comparison of BuChE and its $L^{286}K$ mutant and the BuChE/AChE chimera. In the center panel, a comparison of the natural alternatives of AChE (E6) and AChE (E5), and the chimera. In the right panel, a comparison of representative data for the $D^{70}G$ variant and BuChE. The chimera is presented in the left panel and BuChE in the right panel to assist correlation of the rates of $L^{286}K$ and $D^{70}G$. BuChE inactivations: ■ 1 nM, ◪ 5 nM, ▲ 10 nM, ▼ 50 nM DFP. AChE (E6) inactivations: □ 0.1 μM, △ 0.5 μM, ▽ 1 μM DFP; AChE (E5) ⊠ 1 μM DFP. $L^{286}K$ BuChE inactivation: ◇ 50 nM DFP. $D^{70}G$ inactivation: ●, 5 nM DFP. B: a logarithmic function of the regain in activity vs. time is presented in the left panel for BuChE ■, the BuChE/AChE chimera ◆, and the $L^{286}K$ mutant ◇ in 1 mM PAM; in the center panel for AChE (E6) ▽, AChE (E5) ⊠, and the chimera ◆, in 0.6 mM; and in the right panel, representative data for BuChE, ●, and the $D^{70}G$ variant, ■, in 1 mM PAM.

test whether variations in the C-terminus [28] would affect catalytic properties of isolated, immobilized ChEs, we used ACHE cDNA vectors. These included a vector encoding the major form of human AChE expressed in brain and muscle [26], and designated E6, and an alternative vector, differing in the 3'-terminus and representing the variant ACHE mRNA species expressed in hematopoietic and tumor cells (E5) [8]. Several natural and site-directed mutants of recombinant human BuChE completed this series. These included the 'atypical' $D^{70}G$ variant, alone or in combination with other natural mutations [17,18], and site-directed point mutations at the acyl-binding site [4]. Fig. 2B positions these changes on the ribbon model of human BuChE and Table 1 lists these alterations.

We next wished to examine the kinetic consequences that changing these defined regions in the enzyme would confer on the different steps of catalysis. To this end, each of the recombinant, immobilized enzyme variants was reacted with DFP, followed by regeneration of activity by PAM. DFP and PAM concentrations were chosen to yield phosphorylation and reactivation half-times measured in minutes. The *Xenopus* oocyte-produced enzymes were enriched by adsorption onto an immobilized antibody [11,24]. ELISA measurements provided determinations of the amounts of recombinant proteins for evaluation of k_{cat} values and confirmed that all of the examined proteins were produced in quantities of the same order of magnitude.

3.2. The gorge lining contributes to enzyme phosphorylation

Rates of DFP inactivation were determined for normal BuChE, $L^{286}K$ BuChE and the chimera as compared with the two alternative forms of AChE (Fig. 3A). The second order rate constant for inactivation of AChE by DFP, 7×10^4 min^{-1} M^{-1} (Table 1), was found to be 160-fold lower than that of BuChE. This value was in good agreement with the value of 3×10^4 min^{-1} M^{-1} determined for native, purified human AChE [14] and was almost identical to the 9.1×10^4 min^{-1} M^{-1} value determined more recently for purified recombinant human AChE [19] and to the value of 9.5×10^4 min^{-1} M^{-1} determined by Jandorf et al. [7]. Introduction of a positive charge into the acyl-binding site of the $L^{286}K$ mutant slowed the inactivation rate of BuChE approximately 8-fold (Fig. 3A). In contrast, substitution of the gorge lining by the corresponding AChE peptide with its 27 amino acid replacements, including 12 non-conservative substitutions [13], reduced this rate 60-fold, down to the level displayed by the two AChE forms (Fig. 3A).

In general, inactivation rates of natural BuChE mutants were not severely affected. There was virtually no effect of $D^{70}G$ or $S^{425}P$ alone, but the $D^{70}G/S^{425}P$ double mutant displayed a lowered inactivation rate which was paradoxically restored in the $D^{70}G/S^{425}P/Y^{114}H$ natural triple mutant [4]. Most site-directed acyl-site mutants of L^{286} and F^{329} also displayed 2- to 7-fold moderately lower inactivation rates than native BuChE, with the exception of $F^{329}Q$, which remained unchanged.

3.3. Active site charges hamper reactivation

To study the dephosphorylation step of catalysis, the same series of enzymes that were inhibited by DFP were further assessed for rates of reactivation by PAM. The K_d for DIP-BuChE, 0.3 mM, was close to the value of 0.2 mM found for purified diethylphosphoryl-BuChE [32]. Incidentally, the serum PAM levels that are achieved during therapy for OP poisoning do not exceed 40 μM [33], well below this value.

The pseudo-first order rate constant for reactivation of DFP-inhibited BuChE (Table 1) was 25-fold higher than for either form of AChE (Fig. 3B and Table 1). The chimera displayed a reactivation constant only moderately (4-fold) lower than that of native BuChE, demonstrating that only a small part of the difference in reactivation between AChE and BuChE is attributable to gorge-lining residues. Moreover, the lower rate for AChE and the chimera was due to a lower true first-order rate constant (k_r), whereas the affinity for PAM, K_d, remained unchanged (Table 2). In contrast, the BuChE $L^{286}K$ mutant displayed a 40-fold reduction in the pseudo-first order rate constant for reactivation, reflecting a reduction in both reactivation rate and affinity for PAM, the latter probably influenced by electrostatic repulsion by the positive charge that was introduced. This emphasizes the importance of the acyl-binding site in the reactivation process. We further

Table 2
Constants for PAM reactivation of DIP-ChEs [a]

Variant	$k_r \times 10^3$ (min^{-1})	K_d (mM)
BuChE	220 ± 60 (4)	0.30 ± 0.08
Chimera	48 ± 20 (3)	0.34 ± 0.13
AChE (E6)	7 ± 3 (3)	0.27 ± 0.04
AChE (E5)	10 ± 5 (2)	0.19 ± 0.07
$L^{286}K$	35 ± 27 (3)	> 5

[a] True first order rate constants for reactivation (k_r) and dissociation constants for the DIP-enzyme/PAM complex (K_d) were evaluated from a plot of the reciprocals of the pseudo-first order rate constants vs. the reciprocals of PAM concentrations between 0.1 and 0.6 mM. Numbers of experiments, in parentheses, and standard deviations are shown.

substituted an acidic (aspartate), a basic (arginine), a sulfhydryl (cysteine) and a polar (glutamine) group for F^{329}. Of these, only the sulfhydryl and acidic groups disrupted the reactivation by 10- to 20-fold (Table 1).

For the natural BuChE variants, effects of $D^{70}G$ and $Y^{144}H$ on reactivation were cumulative. The natural substitution of D^{70} by glycine [17] made the resulting variant reactivate at a rate 5-fold lower than that of the wild-type enzyme (Fig. 3B, right; Table 1). Addition of the $Y^{114}H$ or $S^{425}P$ mutation to the $D^{70}G$ mutation resulted in an even slower reactivating enzyme, and a combination of all three mutations in one variant caused the most severe decrease, 40-fold, in the rate of reactivation (Table 1). Thus, reactivation rates, as compared to the parent enzyme, were seriously impaired in certain natural and site-directed mutants of BuChE, more than in the chimera.

3.4. C-terminal variations are innocuous in catalytic terms

Both alternative ACHE DNA forms led to enzymes with similar inactivation rates (Fig. 3A and Table 1), demonstrating that the natural variability in the C-terminus of AChE contributes to the inactivation rate far less than differences in the acyl-binding site and gorge lining of ChEs. Moreover, reactivation, as well, appeared to be unaffected by the natural C-terminus modifications in AChE (Fig. 3B and Table 1). Finally, the kinetic rate constants derived for DFP-inactivation and PAM reactivation of the AChE preparations encoded by the two 3'-variable ACHE DNAs were indistinguishable (Tables 1 and 2), demonstrating that the natural C-terminal variations in AChE are innocuous in catalytic terms.

3.5. Effects of variations on k_{cat}

To investigate the contribution of specific effects of the analogous reactions on catalysis, and thereby place effects on the analogous reactions in a practical context, the consequences of each variation were further evaluated by determining turnover numbers (k_{cat}, Table 1). The k_{cat} for human BuChE as determined by us, 96,000 min^{-1}, was in good agreement with that reported for recombinant mouse BuChE [31]. Effects on k_{cat} in the various mutants were not cumulations of effects on k_i and k'_r, nor are they expected to be, as only the slowest step will be reflected in the catalytic rate. Thus, substitution of L^{286} with a basic residue reduced reactivation 40-fold but led to only a 5-fold reduction in k_{cat} (Table 1). Other replacements at this position, and substitution of the gorge lining in the chimera, had considerably smaller effects on this value. Thus, modest changes in the catalytic rate may mask large variations in the rates of specific steps in the catalytic process of both native and mutant ChEs.

4. Discussion

In order to more fully understand the biological consequences of the diversity of ChEs, we dissected the effects of specific modifications in these enzymes on distinct steps in catalysis. To this end, we employed successive OP phosphorylation and oxime-induced dephosphorylation as steps analogous to the acylation and possibly deacylation reactions of substrate hydrolysis. Adsorption of the recombinant ChEs onto immobilized selective monoclonal antibodies enriched the enzymes, separated the catalytic steps and prevented OP-induced ageing and oxime-dependent acceleration of substrate hydrolysis. The rate constants thus obtained were similar to those published for the purified proteins in solution. This, in turn, suggests wider use of our method for partial purification by adsorption onto antibody-coated wells of microtiter plates and subsequent activity assay. For example, protein phosphorylation and dephosphorylation by kinases and phosphatases, or other transient covalent modifications modulating protein properties, should be readily available for sequential dissection by this technique.

We chose to focus on three peptide regions within the human ChE molecules. These were (a) the gorge lining, known to differ in hydrophobicity between AChE and BuChE [6,29], (b) the acyl-binding site, known to be critical for the substrate specificities of AChE and BuChE [6,31] and (c) the C-terminal peptide in AChE, which is subject to natural variations due to alternative splicing [8,10]. DFP was chosen for comparative inactivation of these ChEs because of the mechanistic similarities of inactivation by DFP to the acylation stage of catalysis. Moreover, being a symmetric compound, DFP can have no inactive stereoisomer. Set against this is the fact that the phosphorus atom has 4 substituents, unlike the carbonyl carbon of substrates, which has 3, so that the substituents inevitably interact with ChEs somewhat differently than does a substrate. In any event, we found the reactivity of AChE toward DFP to be remarkably lower than that of BuChE, in spite of the close similarity of these two enzymes, a phenomenon noted 40 years ago by Jandorf et al. in the compparison of human AChE and equine BuChE [7]; our use of the 'atypical' and the chimeric enzyme suggested that aromatic gorge-lining residues, especially residues Y^{72} and Y^{124}, which participate in the peripheral anionic site [25], contribute significantly toward this difference. Interestingly, even OP agents that are, unlike DFP, designed as anti-AChE poisons, inactivate BuChE and AChE at similar rates [21], suggesting a special vulnerability of BuChE to OPs. Even

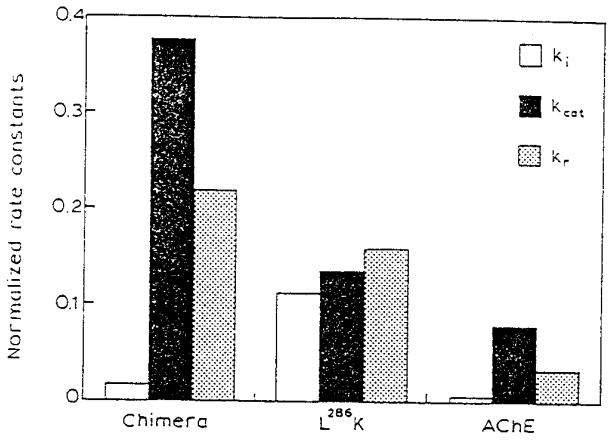


Fig. 4. Tests for the rate-limiting step. Normalized k_i, k_i(mutant)/k_i(BuChE), normalized k_r, k_r(mutant)/k_r(BuChE) and normalized k_{cat}, k_{cat}(mutant)/k_{cat}(BuChE) are presented for each protein. Bar heights for the variants are shown in comparison to the reference enzyme, BuChE.

in the case of those anti-AChEs, BuChE, with a larger binding site, acts as a scavenger to protect AChE [21]. In contrast, the natural alterations of the C-terminus in AChE did not affect its catalytic properties. This further emphasizes the dramatic effects of modifications in the gorge lining and acyl-binding site, regions much more important for interactions with ligands.

To draw conclusions about the effect of mutations on the first phase of catalysis by the chimera, L^{286} mutants and the AChE variants, we compared the changes in their k_i values to those observed in k_{cat}. If acylation by a mutant is rate limiting for catalysis, and if phosphorylation is perfectly analogous to acylation, we would expect a decrease in k_{cat} in the same proportion as in k_i. Thus, k_i(mutant)/k_i(BuChE), called normalized k_i, would be equal to normalized k_{cat}, k_{cat}(mutant)/k_{cat}(BuChE), for any mutant for which acylation is the rate-limiting step. If, however, acylation is not rate limiting for a mutant, there is no coupling of the values of the rate constants. In such cases, the rate-limiting step of catalysis may be seriously impaired while inactivation rate remains high (normalized $k_i \gg$ normalized k_{cat}), or catalysis can remain unaffected while inactivation is impaired (normalized $k_i \ll$ normalized k_{cat}). Fig. 4 presents these normalized values in a bar graph. We find that for the $L^{286}K$ mutant (Fig. 4) acylation appears to be rate limiting (the height of the normalized k_i bar is approximately that for the normalized k_{cat} bar), but for the chimera, normalized k_i is very different from normalized k_{cat}.

In the same way, we can calculate a normalized k_r, k_r(mutant)/k_r(BuChE), to test whether, given the reservations we have noted, reactivation can be a reliable model for deacylation, i.e. whether this parameter gives information that is consistent with findings for the acylation step. For the chimera, the height of the normalized k_r bar was close to that for the normalized k_{cat} bar. Therefore, by the same criterion we use for inactivation/acylation, we can tentatively conclude that for the chimera, deacylation is rate limiting (Fig. 4). This analysis indicates that for the $L^{286}K$ mutant deacylation is also rate limiting (Fig. 4). Thus, for the chimera we find a single rate-limiting step, whereas for the $L^{286}K$ mutant it is in apparent contradiction to our finding regarding acylation. Nevertheless, for the mutant both acylation and deacylation may proceed at closely similar rates, a conclusion previously reached by Quinn on the basis of his mechanistic studies on AChE [20]. Our observation that there are ChEs for which either phase of reaction seems to be rate limiting, apparently indicates the two rates are similar, and that small modification in structure can make either step decisively rate limiting. Finally, the normalized k_r and k_{cat} bars for AChE are approximately equal, implying that for AChE acting on the poor substrate, butyrylthiocholine, deacylation seems to be rate limiting.

The strikingly lower inactivation rate of the chimera than that of BuChE may be attributable to its having its active site gorge narrowed by numerous aromatic residues. (The chimera's acyl binding site is unchanged.) Nevertheless, it appears (Fig. 4) that for the chimera deacylation is the rate-limiting step. This could be due to subtle realignments of functional residues at the catalytic site. For the $L^{286}K$ mutation, both inactivation and reactivation are affected. Introduction of a positive charge at the acyl biding site, and possibly also tightly bound water molecules, may disrupt both phases of the reaction. The capacity of AChE to hydrolyze the disfavored substrate, butyrylthiocholine, is reduced to 1/30th the rate of BuChE. This may indicate that even with a narrower gorge and tighter acyl-binding site, deacylation is rate limiting. With the optimal substrate, we suspect the acylation phase for AChE to be even faster, with deacylation remaining the rate-limiting step.

The accessibility of BuChE as a serum enzyme raises the possibility that PAM acts by regenerating BuChE and allowing it to react with more of the OP before the OP has a chance to inactivate neuromuscular AChE, in effect turning serum BuChE into an OPase. A finding with important consequences for dealing with OP poisoning is that $D^{70}G$, by far the most common of the phenotypically different BuChE variants [34] reacts with DFP as fast as the normal enzyme, but is reactivated by PAM at only one-fifth the rate. Since $D^{70}G$ also has a 4-fold lower specific activity, than the wild type enzyme (ref. [17] and unpublished observations), $D^{70}G$ BuChE will have less detoxifying capacity to protect OP-poisoned individuals than its normal counterpart. Moveover, when intravenous PAM administration, in conjunction with other therapies, is used to treat OP intoxication [30], carriers of $D^{70}G$ BuChE will

have a genetic predisposition to poor responses to such therapy. This applies to 'atypical' homozygotes, as well as heterozygotes, who together compose up to 7.5% of some populations [2]. Thus, the recently reported variable efficacy of PAM treatment [33] may be due to genetic diversity in the treated patients. Individual differences may further be expected in the sensitivity to poisoning by therapeutic anti-ChEs, such as those used in Alzheimer's disease [9]. Natural anti-ChEs, such as glyco-alkaloids in *Solanum* plants [18] and neurotoxins excreted by cyanobacteria [1] may also have diverse effects on these individuals. From an evolutionary point of view, the existence in all vertebrates of two distinct ChEs with specific catalytic properties may be of vital importance as a survival advantage. Moreover, the emergence of genetically polymorphic alleles of BuChE can be advantageous to all higher animal species, which must be able to defend themselves against threats characteristic of the environment to which they have adapted. The biological effects of natural and synthesized anti-ChE agents, depending as they do upon an initial encounter with BuChE, will thus be greatly influenced by just which BuChE allele an individual carries.

Acknowledgements

[28] Numbering of human BuChE residues is according to its published sequence ~~(Prody et al., 1987)~~. Point mutations are indicated by the wild-type residue, the position in the sequence, and the mutant residue; thus, $L^{286}K$ is the replacement of leucine 286 by arginine.

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References

[1] Carmichael, W.W., The toxins of cyanobacteria, *Scientific Am.*, 270 (1994) 64–72.

[2] Ehrlich, G., Ginzberg, D., Loewenstein, Y., Glick, D., Kerem, B., Ben-Ari, S., Zakut, H. and Soreq, H., Population diversity and distinct hapotype frequencies associated with ACHE and BCHE genes of Israeli Jews from transcaucasian Georgia and from Europe, *Genomics*, 22 (1994) 288–295.

[3] Gilson, J.K., Straatsma, T.P., McCammon, J.A., Ripoll, D.R., Faerman, C.H., Axelsen, P.H., Silman, I. and Sussman, J.L. Open 'back door' in a molecular dynamics simulation of acetylcholinesterase, *Science*, 263 (1994) 1276–1278.

[4] Gnatt, A., Loewenstein, Y., Yaron, A., Schwarz, M. and Soreq, H., Site-directed mutagenesis of active site residues reveals plasticity of human butyrylcholinesterase in substrate and inhibitor interactions, *J. Neurochem.*, 62 (1994) 749–755.

[5] Hackley Jr., B.E., Plapinger, R., Stolberg, M. and Wagner-Jauregg, T., Acceleration of the hydrolysis of organic fluorophosphates and fluorophosphonates with hydroxamic acids, *J. Am. Chem. Soc.*, 77 (1955) 3651–3653.

[6] Harel, M., Sussman, J.L., Krejci, E., Bon, S., Chanal, P., Massoulie, J. and Silman, I., Conversion of acetylcholinesterase to butyrylcholinesterase: modeling and mutagenesis, *Proc. Natl. Acad. Sci. USA*, 89 (1992) 10827–10831.

[7] Jandorf, B.J., Michel, H.O., Schaffer, N.K., Egan, R. and Summerson, W.H., The mechanism of reaction between esterases and phosphorus-containing anti-esterases, *Disc. Faraday Soc.*, 20 (1955) 134–142.

[8] Karpel, R., Ben Aziz-Aloya, R., Sternfeld, M., Ehrlich, G., Ginzberg, D., Tarroni, P., Clementi, F., Zakut, H. and Soreq, H., Expression of three alternative acetylcholinesterase messenger RNAs in human tumor cell lines of different tissue origins, *Exp. Cell Res.*, 210 (1994) 268–277.

[9] Knapp, M.J., Knopman, D.S., Solomon, P.R., Pendlebury, W.W., Davis, C.S. and Gracon, S.I., A 30-week randomized controlled trial of high-dose tacrine in patients with Alzheimer's disease, *J. Am. Med. Assn.*, 271 (1994) 985–991.

[10] Li, Y., Camp, S. and Taylor, P., Tissue-specific expression and alternative mRNA processing of the mammalian acetylcholinesterase gene, *J. Biol. Chem.*, 268 (1993) 5790–5797.

[11] Liao, J., Mortensen, V., Norgaard-Pedersen, B., Koch, C. and Brodbeck, U., Monoclonal antibodies against brain acetylcholinesterases which recognize the subunits bearing the hydrophobic anchor, *Eur. J. Biochem.*, 215 (1993) 333–340.

[12] Liu, W., Zhao, K.-Y. and Tsou, C.-L., Reactivation kinetics of diethylphosphoryl acetylcholine esterase, *Eur. J. Biochem.*, 151 (1985) 525–529.

[13] Loewenstein, Y., Gnatt, A., Neville, L.F. and Soreq, H., Chimeric human cholinesterase: identification of interaction sites responsible for recognition of acetyl- or butyrylcholinesterase-specific ligands, *J. Mol. Biol.*, 234 (1993) 289–296.

[14] Main, A.R. and Iverson, F., Measurement of the affinity and phosphorylation constants governing irreversible inhibition of cholinesterases by di-isopropyl phosphofluoridate, *Biochem. J.*, 100 (1966) 525–531.

[15] Massoulie, J., Pezzementi, L., Bon, S., Krejci, E. and Vallette, F.M., Molecular and cellular biology of cholinesterases, *Prog. Neurobiol.*, 41 (1993) 31–91.

[16] McGuire M.C., Nogueira, C.P., Bartels, C.P., Lightstone, H., Hajra, A., van der Spek, A.F.L., Lockridge, O. and La Du, B.N., Identification of the structural mutation responsible for the dicbucaine-resistant (atypical) variant form of human serum cholinesterase, *Proc. Natl. Acad. Sci. USA*, 86 (1989) 953–957.

[17] Neville, L.F., Gnatt, A., Padan, R., Seidman, S. and Soreq, H., Anionic site interactions in human butyrylcholinesterase disrupted by two single point mutations, *J. Biol. Chem.*, 265 (1990) 20735–20738.

[18] Neville, L.F., Gnatt, A., Loewenstein, Y., Seidman, S., Ehrlich, G. and Soreq, H., Intramolecular relationships in cholinesterases revealed by oocyte expression of site-directed and natural variants of human BCHE, *EMBO J.*, 11 (1992) 1641–1649.

[19] Ordentlich, A., Kronman, C., Barak, D., Stein, D., Ariel, N., Marcus, D., Velan, B. and Shafferman, A., Engineering resistance to aging of phosphylated human acetylcholinesterase. Role of hydrogen bond network in the active center, *FEBS Lett.*, 334 (1993) 215–220.

[20] Quinn, D.M., Acetylcholinesterase: enzyme structure, reaction dynamics, and virtual transition states, *Chem. Rev.*, 87 (1987) 955–979.

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[21] Raveh, L., Grunwald, J., Marcus, D., Papier, Y., Cohen, E. and Ashani, Y., Human butyrylcholinesterase as a general prophylactic antidote for nerve agent toxicity, *Biochem. Pharmacol.*, 45 (1993) 2465–2474.

[22] Rosenberry, T.L., Acetylcholinesterase, *Adv. Enzymol.*, 43 (1975) 104–210.

[23] Schumacher, M., Camp, S., Maulet, Y., Newton, M., MacPhee-Quigley, K., Taylor, S.S., Friedmann, T. and Taylor, P., Primary structure of *Torpedo californica* acetylcholinesterase deduced from its cDNA sequence, *Nature*, 319 (1986) 407–409.

[24] Seidman, S., Ben Aziz-Aloya, R., Timberg, R., Loewenstein, Y., Velan, B., Shafferman, A., Liao, J., Norgaard-Pedersen, B., Brodbeck, U. and Soreq, H., Overexpressed monomeric human acetylcholinesterase induces subtle ultrastructural modifications in developing neuromuscular junctions of *Xenopus laevis* embryos, *J. Neurochem.*, 62 (1994) 1670–1681.

[25] Shafferman, S., Velan, B., Ordentlich, A., Kronman, C., Grosfeld, H., Leitner, M., Flashner, Y., Cohen, S., Barak, D. and Ariel, N., Substrate inhibition of acetylcholinesterase: residues affecting signal transduction from the surface to the catalytic center, *EMBO J.*, 11 (1992) 3651–3658.

[26] Soreq, H., Ben-Aziz, R., Prody, C.A., Seidman, S., Gnatt, A., Neville, L., Lieman-Hurwitz, J., Lev-Lehman, E., Ginsberg, D., Lapidot-Lifson, Y. and Zakut, H., Molecular cloning and construction of the coding region of human acetylcholinesterase reveals a G,C-rich attenuating structure, *Proc. Natl. Acad. Sci. USA*, 87 (1990) 9688–9692.

[27] Soreq, H., Gnatt, A., Loewenstein, Y. and Neville, L.F., Excavations into the active site gorge of cholinesterases, *Trends Biochem. Sci.*, 17 (1992) 353–358.

[28] Soreq, H. and Zakut, H., *Human Cholinesterases and Anticholinesterases*, Academic Press, San Diego, 1993.

[29] Sussman, J.L., Harel, M., Frolow, F., Oefner, C., Goldman, A., Toker, L. and Silman, I., Atomic structure of acetylcholinesterase from *Torpedo californica*: a prototypic acetylcholine-binding protein, *Science*, 253 (1991) 872–879.

[30] Taylor, P., Cholinergic agonists, Anticholinesterase agents. In A.G. Gilman, L.S. Goodman, T.W. Rall, A.S. Nies and P. Taylor (Eds.), *The Pharmacological Basis of Therapeutics*, 8th Ed., Pergamon Press, New York, 1990, pp. 122–147.

[31] Vellom, D.C., Radic, Z., Li, Y., Pickering, N.A., Camp, S. and Taylor, P., Amino acid residues controlling acetylcholinesterase and butyrylcholinesterase specificity, *Biochemistry*, 32 (1993) 12–17.

[32] Wang, E.I.C. and Braid, P.E., Oxime reactivation of diethylphosphoryl human serum cholinesterase, *J. Biol. Chem.*, 242 (1967) 2683–2687.

[33] Willems, J.L., DeBisschop, H.C., Verstraete, A.G., Declerck, C., Christiaens, Y., Vanscheeuwyck, P., Buylaert, W.A., Vogelaers, D. and Colardyn, F., Cholinesterase reactivation in organophosphorus poisoned patients depends on the plasma concentrations of the oxime pralidoxime methylsulphate and of the organophosphate, *Arch. Toxicol.*, 67 (1993) 79–84.

[34] Whittaker, M., *Cholinesterase*, Karger, Basel, 1986.

[35] Wilson, I.B., The mechanism of enzyme hydrolysis studied with acetylcholinesterase. In W.D. McElroy and B. Glass (Eds.), *The Mechanism of Enzyme Catalysis*, The Johns Hopkins Press, Baltimore, 1954, pp. 642–657.

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ENGINEERING OF HUMAN CHOLINESTERASES EXPLAINS AND PREDICTS DIVERSE CONSEQUENCES OF ADMINISTRATION OF VARIOUS DRUGS AND POISONS

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The acetylcholine hydrolyzing enzyme, acetylcholinesterase, primarily functions in nerve conduction, yet it appears in several guises, due to tissue-specific expression, alternative mRNA splicing and variable aggregation modes. The closely related enzyme, butyrylcholinesterase, most likely serves as a scavenger of toxins to protect acetylcholine binding proteins. One or both of the cholinesterases probably also plays (a) non-catalytic role(s) as a surface element on cells, to direct intercellular interactions. The two enzymes are subject to inhibition by a wide variety of synthetic (e.g. organophosphorus and carbamate insecticides) and natural (e.g. glycoalkaloids) anticholinesterases that can compromise these functions. Butyrylcholinesterase, may function, as well, to degrade several drugs of interest, notably aspirin, cocaine and cocaine-like local anesthetics. The widespread occurrence of butyrylcholinesterase mutants with modified activity, complicates further this picture, in ways that are only now being dissected through the use of site-directed mutagenesis and heterologous expression of recombinant cholinesterases.
additional key words: genetics/iatrogenic disease

Table of contents

Abbreviations and nomenclature:
ABS, acyl binding site; ACh, acetylcholine; ACHE, acetylcholinesterase gene; AChE, acetylcholinesterase enzyme; AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; AS, active (catalytic) site; ASG, active site gorge; ATCh, acetylthiocholine; BCHE, butyrylcholinesterase gene; BTCh, butyrylthiocholine; BuChE, butyrylcholinesterase enzyme; BW 284C51, 1,5-bis(4-allyldimethylammonium phenyl)pentan-3-1 dibromide; CBS, choline binding site; ChAT, choline-acetyltransferase; CHE, cholinesterase gene; ChE, cholinesterase enzyme; CNS, central nervous system; DFP, diisopropylfluorophosphonate; DIP-, diisopropylfluorophosphoryl; G1, G2, etc. monomeric, dimeric, etc. globular forms of a cholinesterase; GPI, glycophosphatidyl inositol; HD, Huntington's disease; isoOMPA, tetraisopropyl pyrophosphroamide; K_{ss}, inhibition (dissociation) constant for substrate inhibition; mAChR, muscarinic acetylcholine receptor; MEPQ, 7-(methylethoxyphosphinyloxy)-1-methyl-quinolinium iodide; nAChR, nicotinic acetylcholine receptor; NME, neuromuscular endplate; OP, organophosphorus agent; 2-PAM, 2-pyridine aldoxime methiodide; PAS, peripheral anionic site; PD, Parkinson's disease; THA, 1,2,3,4,-tetrahydro-9 aminoacridine (tacrine); WT, wild type.

For identification of amino acid residues, the sequence position in a particular ChE is given, followed in parentheses by the identity and position number of the homologous residue in *Torpedo* AChE.

Acetylcholinesterase (EC 3.1.1.7, AChE) and butyrylcholinesterase (EC 3.1.1.8, BuChE) are two closely homologous proteins. Both are present in all vertebrates, and both are capable of hydrolyzing the neurotransmitter, acetylcholine (ACh). Several earlier reviews, by Taylor (1991), Massoulie *et al.* (1993) and Soreq and Zakut (1993), and one quite recent one by Taylor and Radic (1994) may be consulted for specialized information on sub-topics, especially on cholinesterases (ChEs) of non-human species and on the cell biology aspects of these enzymes. In the present review, we focus on the human genes and their natural and engineered mutations, and the impact of these mutations on the responses of the encoded enzymes to drugs and poisons.

The most obvious and best studied function of AChE is the hydrolysis of ACh to terminate neurotransmission at the neuromuscular junction and nicotinic or muscarinic brain synapses and secretory organs of various sorts. Fig. 1A outlines this catalytic process and presents the analogy between it and the interaction of ChEs with OP inhibitors. AChE is characterized by a narrow specificity for ACh and certain inhibitors and by substrate inhibition. The role of BuChE, beyond hydrolyzing ACh at concentrations that would cause inhibition of AChE (Augustinsson, 1948), has not been identified with certainty, but as it has a wider substrate specificity and interacts with a broader range of inhibitors, it has been proposed that it scavenges anti-ChE agents, protecting synaptic AChE from inhibition and the multitude of ACh receptors from blockade (Soreq *et al.*, 1992).

1. Protein chemistry and enzymology

Functionally and structurally, the ChEs belong to the class of proteins known as the lipase/esterase family. The two subclasses of this family hydrolyze vastly different substrates, the first, in a homogeneous aqueous phase, the other, at lipid-water interfaces. Still, substantial sequence homology between its members has been recognized, predominantly in the N-terminal half of the molecules. However, comparison of the three-dimensional structure of *Geothichum candidum* lipase and *Torpedo* AChE revealed a strikingly similar topology that extends through the whole length of the polypeptide chain. This common topological fold, named the α/β-hydrolase fold, has been identified in all members of the lipase/esterase family and in a number of other unrelated hydrolases with no sequence similarity to the ChEs or to each other (Ollis *et al.*, 1992). Comparison of structurally conserved (Greer, 1990) and variable regions of the different proteins suggests that invariant residues are placed in key internal positions to ensure correct folding and that the majority of low-variability positions are in the core of the protein. In contrast, residues facing the surface are much less conserved. A large β-sheet and crossover helices form the conserved scaffold, whereas loops covering the scaffold and surrounding the active site determine substrate specificity and are consequently unique to each enzyme (Cygler *et al.* 1993).

More specifically, the three dimensional structure of AChE is becoming impressively detailed, thanks to data acquired from X-ray crystallography, combined with the use of specific ligands and site directed mutants. The emerging image is of a globular protein that features an active center buried within its interior. A deep and narrow gorge extending from the surface of the protein down to the active center is the entry to the active site (Sussman *et al.*, 1991). The ChEs are notable for their catalytic rates, which approach the rate of diffusion, the theoretical limit for catalysis by any enzyme. An interesting mechanism has been proposed (Ripoll *et al.*, 1993) to explain this exceptionally high catalytic rate. Computer modeling of the distribution of charges in AChE suggests that the protein is a very strong dipole, with field lines that attract the positively

charged ACh into the gorge and down to the active site where the lowest electrostatic potential lies. Further, the same field will accelerate expulsion of the final product, the acetate ion. It has also been proposed that a mobile flap, a "back door", allows exit of choline from the catalytic site directly to the exterior of the protein without having to travel up the gorge, thus avoiding a potential traffic problem and accelerating catalytic turnover (Gilson *et al.*, 1994). This ingenious proposal awaits experimental verification.

Substrate inhibition studies of AChE suggested that there exists a peripheral anionic site (PAS) (Changeux, 1966). Further evidence for the PAS came from its interaction with the reversible inhibitor, propidium, which did not prevent the simultaneous binding of other inhibitors to the catalytic site (Taylor and Lappi, 1975). Analysis of the amino acid sequences and three dimensional models of AChE has led to further identification of more residues potentially important in PAS involvement in the catalytic activity and inhibitor interaction of AChE. For example, W279 positioned at the entrance to the gorge on *Torpedo* AChE (Fig. 1B). When substituted by alanine, inhibition by the PAS-specific ligand, propidium, was strongly reduced as compared with wild-type *Torpedo* AChE, while action of the catalytic site-specific inhibitor edrophonium was unaffected (Harel *et al.*, 1992). This suggested that W279 is associated with the PAS. Further studies on human AChE confirmed this, and also placed at the PAS, residues Y72 (Y70) and Y124 (Y121) (see abbreviations footnote). Together, these residues close to the entrance to the active site gorge, comprise several overlapping binding sites that all share residues W286 (W279) and D74 (D72) (Barak *et al.*, 1994). The information that the PAS is occupied is thought to be communicated to the active center from D74 (D72) near the surface, on to Y341 (Y334), with which it is hydrogen bonded, and relayed to Y337 (F330) at the hydrophobic subsite of the active center, which may rotate into the active site and block access of potential ligands (Ordentlich *et al.*, 1993a; Shafferman *et al.*, 1992b). Support for the role of D74 (D72) comes from its unique capacity to influence events taking place both at the PAS and the active center (Barak *et al.*, 1994). It was further shown for AChE that W286 (W279) is coupled to W86 in the active center. It was suggested that a "crosstalk" between these two tryptophan residues results in reorientation of W86 (W84) which may interfere with stabilization of enzyme-substrate complexes (Ordentlich *et al.*, 1993a). However, whether or not W286 (W279) is implicated in the well-known phenomenon of substrate inhibition of AChE (Shafferman *et al.*, 1992b; Harel *et al.*, 1992), is controversial. There is no such evidence for a PAS in BuChE, although its substrate activation by ATCh (Cauet *et al.*, 1987) and substrate inhibition by benzoylcholine (Augistinsson, 1948) suggest that one may exist.

Studies on a mutant human BuChE, the "atypical" variant, identified another residue that affects inhibitor interactions of the ChEs. The variation was identified as a point mutation of aspartate 70, which was replaced by a glyicne residue (D70G) (McTiernan *et al.*, 1989; Lockridge, 1990; Neville *et al.*, 1990b), and the variant was demonstrated to display decreased interactions with inhibitors (Gnatt *et al.*, 1990; Neville *et al.*, 1990a; McGuire *et al.*, 1989) and a 4-fold lower specific activity than the wild-type BuChE (Neville *et al.*, 1992). This natural mutation and the many others discovered after it (reviewed by Soreq and Zakut, 1993) are presented in Table 1. The finding of this mutation led to intense work on the homologous residue of AChE (see below).

In the gorge lining six aromatic residues, typical of AChEs, are different in BuChEs. Two of them, F288 and F290, which are positioned at the acyl-binding pocket at the bottom of the gorge, were replaced with their counterparts in BuChE, leucine and valine, respectively. The resultant double mutant hydrolysed

BTCh almost as well as ATCh (Harel *et al.*, 1992). It was also inhibited by the BuChE-specific inhibitor isoOMPA at a rate considerably faster than AChE. Thus, the bulky phenylalanyl residues at the gorge lining in AChE seem to prevent entrance of BTCh and iso-OMPA into the acyl-binding pocket. Similar studies were performed on mouse AChE (Vellom *et al.*, 1993), with parallel results. Table 2 summarizes briefly the many site-directed mutagenesis studies on variable ChEs and their biochemical implications.

A chimeric protein was created by exchanging a polypeptide sequence that includes the rim of the gorge (including D70) with the corresponding sequence from human AChE. It includes part of the conserved gorge lining and oxyanion hole, and the choline binding site of human BuChE (residues 58 to 133 of the BuChE sequence). Therefore, its properties help explain the roles of these sites in catalysis and inhibitor interactions (Loewenstein *et al.*, 1993a). The catalytic properties of the chimera were, in general, identical to those of BuChE, sharing with BuChE recognition of succinyl choline as a substrate and physostigmine (eserine) as an inhibitor. However, the chimera acquired the AChE-like sensitivity to inhibition by echothiophate and iso-OMPA and displayed a pattern of inhibition, more similar to that of AChE than of BuChE, toward the anti-asthma drug, bambuterol, the local anesthetic, dibucaine, and the AChE-specific inhibitor, BW 284C51. It was concluded that the exchanged residues interact with inhibitors but not substrates. A similar chimera, but of the mouse ChEs, was a substitution of the amino-terminal 174 residues of AChE, which probably constitute the lip of the active site gorge, with the cognate residues of BuChE. This chimera showed considerably less inhibition by BW 284C51 than the parent AChE (Vellom *et al.*, 1993). Additional support for the role of this region in inhibitor interactions comes from kinetic studies of DFP inhibition of human ChEs. BuChE was inhibited at a 160-fold faster rate than AChE, and the human BuChE/AChE chimera, which differs from BuChE by only 15 non-conserved residues, was inhibited at a rate similar to that of AChE (Schwarz *et al.*, 1994). In contrast, substitutions at the acyl-binding pocket resulted in a 2- to 5-fold reduction in rate. Also changes at the carboxyl terminus of AChE had no effect on the inactivation rate, re-emphasizing the importance of inhibitor interactions at the same region as was exchanged in the human ChE chimera.

Fig. 2 presents the diverse mutations introduced into ChEs from various species. At the active center of *Torpedo* AChE, site-directed mutagenesis was employed to identify S200, H440 and E327 as the catalytic triad. This is the first serine hydrolase found with glutamate as opposed to aspartate in its charge relay system (Sussman *et al.*, 1991; Gibney *et al.*, 1990; Shafferman *et al.*, 1992a). Subsequently, other members of the lipase/esterase family were found to display similar S-H-E triads. Substitution of active site human W86 (W84) with alanine caused a 660-fold decrease in the affinity for ATCh but had no effect on the affinity for an uncharged isosteric substrate, 3,3-dimethylbutyl thioacetate. Diminished inhibition by PAS-specific agents was also observed. This identified W86 (W84) as the classical "anionic" subsite of the active center that binds the cationic quaternary ammonium group of choline and active site inhibitors such as edrophonium (Ordentlich *et al.*, 1993a). In fact the "anionic" site is a tryptophan residue, the π electrons of which interact with the quarternary ammonium group (Harel *et al.*, 1993). It also reenforces the suggested involvement of this residue in communicating to the active center, the effect of occupancy of the PAS by a ligand (Shafferman *et al.*, 1992b). At the active site of BuChE, alterations at F329 (F331), L286 (F288) and Y440 (Y442) had marked effects on inhibitor interactions. In the case of F329 (F331) and Y440 (Y442) this was achieved without significantly changing K_m, demonstrating distinct requirements for inhibition and substrate interactions at the acyl- and choline-binding pocket

(Gnatt *et al.*, 1994).

Mutation of G199 in *Torpedo* (Gibney *et al.*, 1990) or F297 (F290) in mouse AChE (Vellom *et al.*, 1993) eliminates substrate inhibition, suggesting, again, that the mutations interrupt intramolecular information transfer between the surface and the interior of the protein.

2. The human cholinesterase genes

Structure of the gene

In man, the two functionally distinct ChEs, AChE and BuChE, which share a high degree of amino acid sequence homology (>50%), are encoded by two separate genes, ACHE and BCHE, respectively (Soreq *et al.*, 1990; Fig. 3). The two genes have similar exon-intron organization (see below) but radically different nucleotide composition, ACHE being G,C-rich while BCHE is A,T-rich. The electric eel, *Torpedo marmorata*, has both an ACHE and a BCHE gene (Toutant *et al.*, 1985), but in insects a single gene encodes one ChE protein with mixed AChE and BuChE properties (Hall and Malcolm, 1991; Taylor and Radic, 1994). Gene duplication and divergence into separate AChE and BuChE enzymes apparently occurred before the evolution of the first vertebrates (Soreq and Zakut, 1990; Taylor, 1991). The presence of two distinct CHE genes in all vertebrates studied to date, indicates that both protein products are biologically required in these species, and presumably that they have distinct roles.

The human ACHE gene spans about 7 kb and includes 6 characterized exons and 4 introns. It can, through alternative splicing, give rise to several different mRNA transcripts (Sikorav *et al.*, 1988; Maulet *et al.*, 1990) (see below). The BCHE gene is much larger than ACHE, spanning 70 kb, and consists of 4 exons, the first of which is non-translatable but contains two potential translation initiation sites, and the second of which contains 83% of the coding sequence (Arpagaus *et al.*, 1990; Gnatt *et al.*, 1991). Only one BCHE mRNA transcript has been identified to date.

Chromosomal location

Mapping of the human BCHE gene to its defined chromosomal location, 3q26-ter, was first performed by *in situ* hybridization to lymphocyte chromosomes and by blot hybridization to DNA of hybrid somatic cells (Gnatt *et al.*, 1990). Direct PCR amplification of human BCHE-specific DNA fragments from somatic cell hybrids and chromosome sorted libraries later confirmed this mapping of the BCHE gene to chromosome 3q26-ter (Gnatt *et al.*, 1991). When using ACHE specific primers, a prominent PCR product was observed with DNA from two different cell-lines and from one chromosome sorted library, all containing DNA from human chromosome 7. The use of fluorescent *in situ* hybridization with biotinylated ACHE DNA, mapped the refined position of the ACHE gene to chromosome 7q22 (Ehrlich *et al.*, 1992; Getman *et al.*, 1992). These findings confirmed predictions that the two closely related CHE genes are not genetically linked in the human genome (Gnatt *et al.*, 1991). They further revealed that these two apparently unrelated genes are both located at chromosomal sites subject to frequent breakage in leukemias (Ehrlich *et al.*, 1994).

Control elements

In man, AChE is produced in muscle and nerve, in hemopoietic cells (Patinkin *et al.*, 1990; Lev-Lehman *et al.*, 1994; Soreq *et al.*, 1994), embryonic tissues (Zakut *et al.*, 1985; Zakut *et al.*, 1990), several tumors (Lapidot-Lifson *et al.*, 1989) and germ cells (Malinger *et al.*, 1989). Since the gene encoding AChE exists as a single copy, the regulatory elements controlling its activity are expected to respond to activating factors specific for these tissues and cell types. Indeed,

a 596 bp fragment upstream of the initiation site for transcription contains numerous consensus motifs characteristic of binding sites for various transcription factors (Ben Aziz-Aloya *et al.*, 1993). The observed sequences include several types of nerve-specific motifs: the Egr-1 sequence, characteristic of brain-specific signal transduction pathways, the CBEB motif, that predicts cAMP responsiveness, and the AP2 signal, that is unique to genes expressed in neuronal crest cells. In addition, several MyoD motifs, associated with muscle-specific gene expression and the E-box motif found in the hematopoietically expressed heavy chain immunoglobulin genes are present. The GAGA, Zeste and USF motifs, known as recognition sites for genes induced during embryonic development were also observed (Ben Aziz-Aloya *et al.*, 1993). Thus, the tightly regulated yet pleiotropic expression of ACHE in humans, could at least in part stem from the activity of the regulatory elements present in this short sequence. However, the promoter sequence alone cannot explain the intricate pattern of tissue-specific expression shown by the ACHE gene, as is evident from the observation that in transgenic mice carrying this promoter sequence and the human AChE coding sequence, AChE production was found to be limited to the central nervous system (CNS) (Andres *et al.*, 1994). This suggests that additional control elements, upstream from the sequenced promoter and which were not included in the sequence incorporated into the genome of the transgenic mice, are involved in regulating AChE production.

Coordination of expression with other cholinergic factors

Several mechanisms ensure that there is an appropriate balance of the proteins that function at the cholinergic synapse. The ACh transporter is encoded by an intron-less sequence embedded in the choline acetyltransferase (ChAT) gene, so expression of the two is coordinated (Erickson *et al.*, 1994). Expression of the acetylcholine receptor (AChR), too, is coordinated with that of AChE (Kristt and Kasper, 1983), in spite of the fact that it is the product of several genes, located on different chromosomes. The molecular mechanism of this coordination is unknown, but increasing the amount of AChE expressed at the synapse by injection of ACHE mRNA into fertilized *Xenopus* eggs, causes morphological changes in neuromuscular junctions within the resultant embryos (Seidman *et al.*, 1994). These are particularly conspicuous in electron micrographs of myotomes, where the synapse covers a larger surface area and the cleft is wider, seemingly coordinated with the increased amount of the nicotinic ACh receptor (nAChR) (Shapira *et al.*, 1994).

Alternative splicing

Yet another level of control over expression of the ACHE gene is that of alternative splicing, a process involving the precise excision of intronic sequences from the precursor nuclear mRNA. All AChE mRNA forms include exons 1 to 4, and alternative splicing may occur both at their 5' and their 3' ends. In the major mRNA form, found mainly in brain and muscle, the E4 exon is directly continued with E6, the connecting sequence, including I4 and E5, being spliced out (Fig. 3). Two alternatively spliced forms encode a protein with the potential to be anchored through a glycophosphoinositide (GPI) residue to the cell surface of hematopoietic cells; these two alternative transcripts are also expressed in several different tumor cell lines (Karpel *et al.*, 1994a). In one of these mRNA forms, the transcript continues through I4, E5 and E6; in the other, I4 is spliced out. An open reading frame continues through E4, I4 and a small part of E5, is missing E6 but encodes a 29 residue hydrophobic polypeptide sequence typical of sequences that can be enzymatically exchanged for a GPI membrane anchor (Turner, 1994). Therefore, at least three forms of human AChE mRNA are predicted, differing in the 3'-ends of their coding sequences. The major brain and muscle form contains E6, whereas those encoding the two potentially

GPI-linked proteins include the open reading frames of I4 and E5, or E5 alone (Karpel *et al.*, 1994a). The alternative splicing patterns at the 5'-end of human ACHE mRNA have not yet been fully characterized; however, it is clear that they do not change the coding sequence.

Polymorphism of molecular forms

For AChE, several post-translational modifications including palmitoylation (Roberts *et al.*, 1988a), oligomeric assembly, association with noncatalytic subunits, glycosylation and the attachment of phospholipids have been discovered, some of which affect the quaternary structure of the enzyme or direct it to its correct subcellular localization (Massoulie *et al.*, 1993).

The ChEs are largely extracellular, being either soluble or attached to the external surfaces of cells. Monomers of the AChE molecule may associate to form dimers and higher levels of organization through inter-monomer disulfide bonds and disulfide bonds to a non-catalytic polymer. These forms are referred to as G1 (globular, monomeric), G2 (disulfide-linked dimers of G1) and G4 (non-covalently associated dimers of G2). G4s may be linked in mammalian brain to a 20 kDa polymer of undefined nature (Inestrosa *et al.*, 1987; Fuentes *et al.*, 1988), or to a GPI which is anchored in the plasma membrane of cells, notably erythrocytes and cells of the *Torpedo* electric organ (Futerman *et al.*, 1985a,b; Silman and Futerman, 1987; Taylor, 1991). However, in rat brain a hydrophobic region of AChE has been identified through which the enzyme can directly bind to membranes, and no 20 kDa protein associated with AChE was observed in this study (Andres *et al.*, 1990, 1992). One to 3 G4s may be linked to ends of the 3 strands of a collagen-like protein, achieving yet higher levels of assembly (Hall, 1973). There is good evidence that the forms associated with the collagen-like tail are bound to heparan sulfate proteolglycans of the basal lamina of neuromuscular junctions and muscle endplates (McMahan *et al.*, 1978). The carbohydrate composition of membrane bound forms of AChE from brain and erythrocytes varies, as seen by interactions with lectins, suggesting an additional level of complexity of ChE polymorphism (Meflah *et al.*, 1984). AChE also appears in a soluble form in serum, and hybrids of AChE and BuChE have also been reported (Tsim *et al.*, 1988).

BuChE appears in many of the same forms (G1, G2, G4) as AChE. G1 is also disulfide-linked to albumin and another unidentified protein (Masson, 1991). Further experimental work will be required to clarify the molecular basis of the differences between salt-soluble (hydrophilic) and detergent-soluble (hydrophobic) forms of ChEs, the nature of the 20 kDa mammalian polymer and its collagen-like counterpart in neuromuscular junctions and the cellular site at which these assemblies are made (Massoulie *et al.*, 1993). Further information is likely to come from investigations into the heritable failure to link G4 AChE with its collagen-like tail and the consequent absence of AChE activity at motor endplates (see section 7).

That the oligomeric assembly of BuChE is regulated by tissue-specifically expressed proteins, was suggested by several lines of evidence. *In vitro* transcribed BuChE mRNA, when microinjected alone into *Xenopus* oocytes, induced the formation primarily of dimeric BuChE. However, tissue-extracted mRNA induced higher levels of subunit assembly, as seen when synthetic BuChE mRNA was complemented with muscle poly(A)$^+$ RNA. This resulted in a quantitative distribution of BuChE among several forms that resembled the distribution of muscle AChE (Dreyfus *et al.*, 1989; Soreq *et al.*, 1989). More recently, it was shown that human AChE cannot assemble into multimeric forms in transiently transgenic *Xenopus* embryos (Seidman *et al.*, 1994), although it assembles correctly when expressed in brain neurons of a stably transgenic mouse pedigree

(Andres *et al.*, 1994). These findings suggest that the yet unidentified factors required for AChE assembly direct aggregation in a tissue- or species-specific manner.

Evidence for the importance of glycosylation for the intracellular transport of ChEs, but not for their catalytic activity, comes from experiments in which glycosylation of human BuChE was inhibited by tunicamycin (Dreyfus *et al.*, 1989; Soreq *et al.*, 1989) and in which the glycosylation sites of human AChE at N265, N350 and N464 were mutated (Velan *et al.*, 1993). Similarly, active non-glycosylated recombinant AChE can be produced in bacteria (Fisher *et al.*, 1993).

3. Inhibition of cholinesterases, mechanism and consequences

Inhibition of ChE can be achieved by several different mechanisms. Simple competitive inhibition is caused by such quaternary compounds as edrophonium, which binds selectively to the active site where it is stabilized by interaction of its quaternary nitrogen with the choline-binding pocket, and by hydrogen bonding (Sussman *et al.*, 1992; Harel *et al.*, 1993). In contrast to edrophonium, carbamyl esters serve as hemi-substrates. During catalysis, a carbamoyl enzyme intermediate is formed, which is far more stable than the acetyl-ChE intermediate. The very slow hydrolysis of the intermediate effectively sequesters ChE for several hours. Neostigmine (Fig. 4), one of many physostigmine derivatives, has increased stability and potency equal to or greater than physostigmine. Demecarium, two neostigmine molecules linked by a 10-carbon chain, has even greater affinity, since it interacts not only with the active site but also with the PAS at the entrance of the active site gorge. As an ACh analog, physostigmine can also block nAChRs (Shaw *et al.*, 1985; Coleman *et al.*, 1987). In fact, quaternary ammonium anti-ChE compounds have additional direct actions at cholinergic sites, either as agonists or antagonists. For example, neostigmine affects the spinal cord and the neuromuscular junction, both by inhibition of AChE activity and by stimulation of cholinergic receptors.

ACh is released from motor neurons in fairly well-defined quanta of about 10,000 molecules in response to closely-timed, discrete nerve impulses. Once released, ACh causes a localized depolarization of the muscle fiber membrane, initiating an action potential that is propagated along its length. Normally, the time required for AChE to hydrolyze the ACh released at a single time is shorter than the decay of this endplate depolarization (and the following refractory period). Therefore, each nerve impulse gives rise to only a single wave of depolarization. However, inhibition of AChE will prolong the period in which ACh is available to act on the muscle ACh receptors. This results in a longer decay of the endplate potential, and an overlap with subsequent nerve impulses and muscle fiber depolarizations. This abolition of the synchronization of nerve impulses, endplate depolarizations and action potentials is seen as muscle spasms. Further inhibition of AChE will subsequently cause blockade owing to continuous depolarization.

Organophosphates as anti-cholinesterases

Organophosphates (OPs), mainly man-made but also in at least one example, occurring naturally in cyanobacteria (Carmichael, 1994), act as hemi-substrates of ChEs, specifically phosphorylating the active site serine, just as the natural substrate acylates it (Fig. 5). Since the rate of hydrolysis of the phosphoryl or phosphonyl enzyme is very much slower than deacylation, OPs are effectively irreversible ChE inhibitors.

Because OP poisoning has been recently reviewed (Marrs, 1993), we will only briefly summarize some of the salient points. OPs have also been developed as

chemical weapon systems, and these potential battlefield threats have provoked considerable study of their short- and long-term physiological effects. OP anti-ChEs are potent insecticides, due to their inhibition of the insects' flight muscle ChE, with resulting paralysis and death. Because the OPs are environmentally non-persistent -- being subject to non-enzymatic hydrolysis -- they are increasingly replacing organic chloride compounds which are in disfavor because of their indiscriminate effects (WHO, 1986a,b). As a result of the extensive use of OP pesticides in agriculture, accidental poisoning of humans increased between 1973 and 1984, from half a million to one million cases per year, worldwide (United Nations Security Council, 1984). Particularly affected are locales where their use is poorly regulated. There are immediate effects of OP poisoning, including respiratory depression, muscular paralysis and convulsions (Foutz *et al.*, 1987), and delayed effects including diarrhea, weight loss, insomnia, myopathy and mental depression (Wecker *et al.*, 1978). Although most modern insecticides are designed to have low vertebrate toxicity, subacute dietary consumption of these poisons (contaminating remnants on vegetables and fruits) may induce chronic cholinergic poisoning of fish and animals (Salte *et al.*, 1987), including humans (Ratner *et al.*, 1983). One very serious effect of exposure to OPs is the increased risk of leukemia (Brown *et al.*, 1990). Also, an individual with exceedingly low BuChE activity and a history of exposure to agricultural OPs had a 100-fold amplification of the "atypical" variant BCHE gene, which was not seen in his parents, but was passed on to the next generation (Prody *et al.*, 1989). The clinical consequences of this are not yet apparent, but any potential effect of OPs on the genome is of great concern. A molecular description of some secondary effects of OP poisoning on the nervous system has been presented: the down-regulation of muscarinic receptors following chronic inhibition of AChE (Olianas *et al.*, 1984; Clement, 1991).

Generally, anti-AChE agents at low concentrations cause spontaneous action in the autonomic ganglia, whereas higher concentrations will bring about persistent depolarization via nAChRs, leading to blockade of the ganglia. A similar picture may be drawn for the CNS (Taylor, 1990). The characteristic pharmacological effects of ChE inhibitors are due primarily to the prevention of ACh hydrolysis by AChE at sites of cholinergic transmission, the consequences of which have been described above. However, anti-AChE agents are used to treat glaucoma by reducing intraocular pressure, and to enhance gastrointestinal motility and gastric secretion, via the vagus nerve (Taylor, 1990).

Treatment of OP poisoning

Treatment of OP poisoning includes prophylactic and therapeutic approaches such as protection against the OP agent with reversible inhibitors (Wills, 1970), e.g. pyridostigmine (Fig. 4), which protects some AChE molecules from inactivation by the OP agent, later to spontaneously regenerate a free active enzyme. Muscarinic symptoms, e.g. increased tracheobronchial and salivary secretion, can be effectively antagonized by a sufficient dosage of atropine, an antagonist of the muscarinic receptor (mAChR), while it has virtually no effect on peripheral neuromuscular activation and subsequent paralysis. The catalytically inactive phosphoryl-enzyme can be reactivated by a cationic oxime through nucleophilic displacement of the phosphoryl moiety from the active site serine (Aldridge and Reiner, 1972; see Fig. 1A). Since reactivation by oximes is most marked at the skeletal neuromuscular junctions, it is an important complement to atropine therapy. These beneficial effects are less evident at autosomal effector sites and insignificant in the CNS. 2-PAM is such an oxime, with features of ACh that permit it to bind to the active site of ChEs.

The phosphoryl-enzyme can undergo a rather rapid process of "aging" which leaves

it refractory to reactivation by oximes. This process is probably due to dealkylation, leaving a much more stable aged OP-conjugate (Aldridge, 1975). The ageing process proceeds at a higher rate with phosphonates containing tertiary alkoxy groups than with secondary or primary ones (Aldridge, 1975). E199 in *Torpedo* AChE (Saxena *et al.*, 1993) and two residues in human AChE, the active center E202 (E199) and the peripheral E450 (E443) (Ordentlich *et al.*, 1993b), have been implicated in the aging process. When substituted with glutamine or alanine, respectively, the resulting enzyme variants showed striking resistance to aging following inhibition by DFP (Ordentlich *et al.*, 1993b; see table 1 for details).

Natural ChE poisons

Over a hundred years ago the Western world became aware of the pharmacological properties of calabar bean extracts (Silver, 1974). These were eventually attributed to the ability of physostigmine to inhibit ChEs. The mode of inhibition has been described, above. Synthetic versions, neostigmine and pyridostigmine (Fig. 4) have been made in order to enhance effectiveness or specificity.

Glycoalkaloids and aglycones of the *Solanaceae* are also inhibitors of ChEs. The *Solanaceae* include such important foods as the potato, tomato and eggplant. Although both *in vitro* effects of these substances and cases of poisoning by them have been documented, it is not yet clear whether they exert an evolutionary pressure (Ehrlich *et al.*, 1994).

Potential strategies for improved protection against OPs

An experimental approach to protecting synaptic AChE from OP poisoning has been to augment the existing function of erythrocyte membrane AChE to act as a scavenger of OPs, by injecting human AChE into test animals and monitoring the biological effects of OPs. In test animals the half-life of native exogenous AChE is on the order of 40 h. In such studies (Raveh *et al.*, 1989; Maxwell *et al.*, 1992; Wolfe *et al.*, 1994) exogenous AChE did indeed increase the LD_{50} for soman and MEPQ. A further refinement has been to test human BuChE, the most prevalent soluble circulating ChE (Ashani *et al.*, 1991; Raveh *et al.*, 1993), as a protective agent for mice and rats, and BuChE from bovine fetal or equine serum (Doctor *et al.* 1991; Wolfe *et al.*, 1994; Doctor *et al.*, 1993) in primates. These protocols, too, were successful in protecting against subsequent injections of soman, preventing both acute effects, and long-term (6 weeks) behavioral effects. This approach is claimed (Wolfe *et al.*, 1994) to be much more successful than the established alternative therapy of 2-PAM and atropine, combined with diazepam (to deal with the problem of seizures). However, the combined use of 2-PAM and human BuChE together is potentially more efficacious than either one alone, since phosphoryl-BuChE is rapidly reactivated by 2-PAM, effectively allowing BuChE to catalytically turnover OPs (Schwarz *et al.*, 1994). As a very limited range of OPs has been tested in this manner, it remains to be seen just how effective this strategy will be when applied to cases of accidental exposure to the more sophisticated pesticide OPs or of overdose of pharmacological OPs. For instance, it is known that horse serum BuChE, like "atypical" human BuChE, will not hydrolyze succinyl choline (Whittaker, 1986). Perhaps this signals a limitation on the use of the horse serum ChE as a model for human BuChE. An alternative approach might be to enhance the endogenous production of the body's own AChE and BuChE by taking advantage of characteristics of the corresponding promoters, as they become known.

In light of our still incomplete knowledge of the biology of the ChEs, it is already evident that the use of anti-ChE agents in agriculture and medicine can

have consequences beyond those imagined when these agents were thought to affect only synaptic AChE. The presence of ChEs in such a great variety of tissues and developmental stages in a tightly regulated fashion warns us that indiscriminate interference with these enzymes is likely to have unanticipated effects. Now that these additional roles of ChEs are being elucidated, especially the tissue-specific occurrence of the various types of ChEs, opportunities arise for the development of very selective anti-ChE agents that can take advantage of these properties for the treatment of diseases that themselves display a cell- or tissue-specific pattern of defects.

4. Drugs that are hydrolyzed or scavenged by cholinesterases

When a drug enters the body through the blood stream, its first encounters with a ChE are with AChE of the erythrocyte membrane outer surface and with circulating BuChE. However, since BuChE is capable of interacting with a wider range of ligands than AChE, and in some cases, at much higher rates (Schwarz *et al.*, 1994), it seems likely to be the major scavenger of anti-ChE agents. Support for this can be found in the absence of a correlation between IC_{50} values of AChE for a range of carbamate anti-ChE's, and their LD_{50} values, while a positive correlation between these parameters exists for BuChE (Loewenstein *et al.*, 1993b). Also, there are individuals who carry a variant BCHE allele, one of those that code for catalytically inactive BuChEs, and these people have an increased sensitivity to OP-poisoning (Prody *et al.*, 1989).

Succinyl choline, an inhibitor of AChE, is a commonly used muscle relaxant. BuChE recognizes succinyl choline as a substrate, and the slow hydrolysis of the agent limits the duration of its action *in vivo*. However, a common natural mutant of BuChE, the "atypical" variant, is unable to hydrolyze succinyl choline (see section 7).

BuChE has been shown *in vitro* to hydrolyze the methyl ester bond of cocaine and its derivatives (Isenschmid *et al.*, 1989; Gatley, 1991). *In vivo*, in contrast to cytochrome P-450-catalyzed destruction of cocaine (Fig. 4), which produces hepatotoxic norcocaine nitroxide, BuChE-catalyzed hydrolysis of cocaine generates innocuous products. The serum levels of BuChE, too, as modulated by ChE inhibitors, correlate with serum levels of cocaine and related narcotics (Kambam *et al.*, 1992) and with their physiological effects (Kambam *et al.*, 1993). This clearly indicates a role for BuChE that must be recognized, especially by those practicing in areas or populations of narcotic usage. Furthermore, exogenous (human) BuChE has been shown to confer protection against cocaine toxicity in rats, both when given prophylactically or therapeutically (Dretchen *et al.*, 1992). Following the elucidation of the pharmacological effects of cocaine, a series of analogs was synthesized, yielding some of the local anesthetics still in use today. As their chemistry is based on that of cocaine, not surprisingly they, too, are subject to hydrolysis and inactivation by BuChE (Baldessarini, 1990).

An aryl acylamidase activity of BuChE, which is strongly inhibited by classical cholinesterase inhibitors, has been reported. This may have implications for the hydrolysis of analgesics such as paracetamol (Fig. 4) (Balasubramanian and Bhanumathy, 1993).

5. Neurodegenerative diseases related to cholinergic malfunction

Defective cholinergic signaling has been found in a number of neurodegenerative disorders in which pathological changes in the levels of AChE and BuChE as well as ChAT and AChR are observed (Rakonczay and Brimijoin, 1988). As these symptoms are organ-specific, rather than global, they may indicate a failure of normal tissue-specific post-transcriptional (alternative splicing) or post-translational

modifications. Following is a brief summary of these neurological diseases.

Alzheimer's Disease (AD) is the most common type of adult-onset dementia. It is associated with a progressive and irreversible loss of memory and cognitive functions proceeding for years. The general malfunction of the cholinergic regions of the brain invariably leads to death. Definitive diagnosis is made by post-mortem demonstration of the histopathological hallmarks of the disease -- neuritic plaques (abnormal axonal terminals associated with loss of ACh secretory granules and increased amyloid deposits) and neurofibrillary tangles (formed from thickening of intracellular neurofibrils). There seems to be a correlation between the impairment of cognitive function with an increase in the number of neuritic plaques and neurofibrillary tangles in the hippocampus, amygdala and cerebral cortex (Coyle *et al.*, 1983; Mullan and Crawford, 1993). Moveover, the severity of the disease parallels the reduction in levels of AChE and ChAT in the frontal and temporal cortices (Perry *et al.*, 1978). A diminished number of cholinergic neurons in basal forebrain nuclei and decreased ACh production in the brain of AD patients, are thought to cause some of the characteristic cognitive impairments. However, whether the damage caused to cholinergic innervation is a cause or a result of the pathological changes, is not yet clear. In the affected brain regions, the decrease of AChE is most pronounced in the G4 form (Atack *et al.*, 1983). This loss is accompanied by an increase in BuChE (Atack *et al.*, 1986) and is correlated with selective degeneration of the presynaptic structures (De Kosky and Scheff, 1990). The monomeric globular form (G1) of AChE is assumed to be associated with the postsynaptic structures (Fishman, 1986) and shows no changes in AD, consistent with the absence of postsynaptic pathology. Wright *et al.* (1993) report that the AChE associated with neurofibrillary tangles and amyloid plaques of AD, is differently affected by a variety of inhibitors than is the enzyme of normal axons and cell bodies. Furthermore, several protease inhibitors decrease the cytological staining of AChE in tissues from AD subjects (Wright *et al.*, 1993), raising the possibility that extracellular proteolysis affects AChE activity, specifically or generally, normally or pathologically.

Three broad strategies have been considered in dementia therapy: (1) replacement of lost neurotransmitter, (2) trophic support to slow the degeneration of the nervous system, and (3) intervention, for example prevention of the local inflammatory response thought to be caused by complement activation in the amyloid plaques (Davis *et al.*, 1993). It has been suggested that anti-cholinergic drugs impair the memory of healthy individuals in a manner parallel to that observed early in the development of AD. Therefore, the principal current AD therapeutic approach, and the most promising one in the short term, is the stimulation of the cholinergic system. Precursor loading, with choline or phosphatidyl choline, is ineffective. However, the anti-ChE agent, physostigmine, has been shown to have a small, short-term positive effect on cognitive functions (Davis *et al.*, 1993 and papers therein cited). More recently developed compounds, like SDZ ENA 713 (Sandoz), have greater central selectivity and longer duration of action than physostigmine (Enz *et al.*, 1993) and is thought to bind specifically to the G1 form of AChE, presumed to be the form involved in postsynaptic ACh hydrolysis (Marquis and Fishman, 1985). Its action is concluded to be on the CNS because of its ability to increase the frequency of rapid eye movements during REM sleep (Enz *et al.*, 1991). Nevertheless, higher doses of the drug caused a transient drop in serum BuChE activity, indicating that it inhibits BuChE as well as AChE. When and if it is tested on larger groups, it may be found that individuals with variant BuChEs, especially homozygotes, are more sensitive to the drug than those with the common BuChE.

The first anti-ChE drug to be approved for use in AD therapy in the USA is

Cognex® (Parke-Davis, 1,2,3,4-tetrahydro-9 aminoacridine, THA, tacrine). Recently, there has been a report of a multi-center, double-blind, placebo-controlled trial of THA therapy, which included 663 patients suffering from mild to moderate AD. It was shown that THA produced statistically significant, dose-related improvements. However, after 30 weeks, significant data were available from only 263 patients. The primary reason for withdrawal of patients from the study was asymptomatic hepatotoxicity, as revealed by elevated serum levels of alanine aminotransferase. The susceptibility to THA was highly variable, the level of the aminotransferase usually being less than three times the upper limit of normal value, but in 2% of the cases it reached 20 times the upper limit of normal, *prima facia* evidence of hepatocellular necrosis. The adverse effects were rapidly reversed when treatment was terminated, and the majority of patients were able to return to the study with lower dosages of THA (Knapp *et al.*, 1994; Watkins *et al.*, 1994). The practical benefits of THA therapy have, however, been questioned. It is argued that the improvement of cognitive function in AD patients receiving THA was superficial; the underlying deterioration continued unabated, as became evident when THA therapy was discontinued. Moreover, severe cholinergic side-effects were observed in a significant number (over 10%) of THA treated patients. Finally, only patients suffering from mild to moderate AD respond to THA treatment, while more severe cases do not benefit from this therapy (Winker, 1994). A smaller study (Minthon *et al.*, 1993) has made similar findings. In view of the evidence of the involvement of ChEs in cellular development, and reports of the effect of anti-ChE agents and of altered expression of ChEs on hematopoietic cell differentiation, it is remarkable that the clinical trials of these agents did not investigate effects on the hematopoietic systems of participating subjects.

Other anti-ChE agents are in testing or are advancing toward it (Davis *et al.*, 1993), and research is intense to develop more specific anti-ChE agents. The 1-hydroxy metabolite of THA has entered multi-centered clinical trials in AD. In a study of a series of chloro-substituted THA analogs the 6-chloro substituted THA-derivative was found to have an IC_{50} of 1.8 nM, one of the most potent reversible anti-AChE agents known (Gregor *et al.*, 1992).

Parkinson's Disease (PD) is a common type of adult-onset chronic degenerative disorder of the CNS. Since PD is associated mainly with the dopaminergic, and not with the cholinergic system, few characterizations of AChE in PD have been performed. However, AChE activity has been observed in dopaminergic brain areas and decreased AChE activity and molecular form changes that parallel those found in AD have been observed in up to 30% of PD. Therefore, cholinergic signaling may be connected with neurodegenerative processes in general, or more specifically with the pathophysiology of PD (Ruberg *et al.*, 1986). PD patients are usually treated with tricyclic anti-depressants. The anti-cholinergic side effect of these drugs may be the basis of some of the benefit shown by this treatment of PD patients. Similarly, there is evidence of worsening of the symptoms in a PD-patient receiving the anti-ChE, THA (Ott and Lannon, 1992).

Huntington's Disease (HD) is a dominant inherited autosomal neurodegenerative disorder with symptoms usually evident at the age of 30 to 40, and is associated with genetically programmed cell death in the CNS. The disease, progressing over a 10-20 year period, eventually destroying all motor function. In most HD cases, progressive dementia is a feature when cholinergic neurons of the brain stem are affected. AChE activity is diminished only in selective bundles of the affected area, known for their rich AChE activity and ChAT activity is decreased in these same areas.

Amyotrophic lateral sclerosis (ALS), or motor neuron disease, is characterized by motor neuron degeneration and progressive failure of neuromuscular transmission. Both upper and lower motor neurons are affected. In neuromuscular endplates (NMEs) significant decreases of all forms of AChE is observed. The defect is thought to be related to disassembly of the synapses and NMEs due to an excitotoxin, a failure of a trophic factor, or a failure to detoxify a xenobiotic, the consequent decrease in nerve signaling, causing a defect in AChE excretion (Goonetilleke *et al.*, 1994). The connection between the recent finding that a mutant human superoxide dismutase introduced into test animals can cause the symptoms of ALS (Gurney, *et al.*, 1994), and the observed neural defects has yet to be found.

Myasthenia gravis

Myostigmine, a synthetic physostigmine derivative, is commonly used to treat patients with myasthenia gravis, a neuromuscular disease of an autoimmune nature, characterized by muscle weakness due to autoimmune anti-nAChR antibodies. Edrophonium induces an immediate, but brief, relief of the characteristic symptoms, due to reversible binding to the active site, terminated by rapid excretion of the drug by the kidneys.

There has been noted a considerable individual variation in the dosages of anti-ChE agents required to control the disorder. This may be due to individual levels of the autoantibodies, or may be due to genetic differences in the BuChEs (see section 7).

Hematological diseases

There is ample evidence for perturbations in cholinergic functions being associated with hematological disorders. The increased risk of leukemia following exposure to OP agents has been mentioned. Down's syndrome, like familial Alzheimer's disease, linked to chromosome 21, (Percy *et al.*, 1993), is associated with AChE deficiencies, and affected individuals have a high incidence of leukemia. Paroxysmal nocturnal hemoglobinuria, also associated with an elevated risk of leukemia, is characterized by a failure of the post-translational glycosylation of AChE. This prevents transport to and interaction of the enzyme with the erythrocyte membrane (Turner, 1994). Several other hematological disorders associated with the cholinergic system were described by Soreq and Zakut (1993). Recently, antisense inhibition of ACHE gene expression, using phosphorothioated oligodeoxynucleotides, has been shown to induce massive proliferation of meloid cells in bone marrow cultures, an *ex vivo* mimic of a leukemic syndrome (Soreq *et al.*, 1994). This is a warning that supreme caution must be used in the development and use of anti-ChE drugs or insecticides.

6. Non-CNS excitatory roles

Cytochemical staining has been used extensively to search for specific ChE activities in different tissues during development. AChE expression has been visualized by immunocytochemical or activity-dependent histological methods, and, more recently by *in situ* hybridization. This revealed AChE association with numerous regions of the brain corresponding with the spatial distribution of different components of both cholinergic and non-cholinergic brain regions (Landwehrmeyer *et al.*, 1994). Transient AChE staining was also shown in the medosomal nucleus of the thalamus and its connection in the developing rodent, human and monkey brains (Kostovic and Goldman-Rakic, 1983; Kristt, 1983; Robertson, 1993). Other, distinct neurons in these same areas were positively stained for BuCHE, but not AChE or ChAT activities (Graybiel and Ragsdale, 1982). Thus, several lines of evidence suggest a non-cholinergic role of ChEs, possibly related to morphogenesis.

In the peripheral nervous system cholinergic signaling has been implicated in pancreatic function, as shown by the decreased AChE activity in the intrapancreatic ganglia of rat subject to celiac and superior mesenteric ganglionectomy (Anglade, 1987). In fact, it was the investigation of the turnover of pancreatic phospholipids accompanying cholinergic stimulation of amylase secretion (Hokin and Hokin, 1953), which led eventually to the discovery of the phosphatidylinositol-derived second messengers of neurotransmitter/hormone action.

ChEs can be found in a wide range of tissues and cell types of clinical significance other than muscle fibre and neurons, among them erythrocytes (Silver, 1974), erythroleukemia cell lines (Ajmar, 1983; Bianchi Searra *et al.*, 1986; Karpel *et al.* 1994a), the adrenal medulla (Massoulie and Bon, 1982), ovarian follicles (Karnovsky and Roots, 1964), megakaryocytes (Burstein *et al.*, 1980; Patinkin *et al.*, 1990, 1994) and chorionic villi (Zakut *et al.*, 1991). In addition, ChE activity has further been reported in a number of embryonic tissues derived from ectoderm, mesoderm and endoderm, as well as in various neoplastic tissues such as ovarian carcinomas (Drews, 1975; Zakut et al, 1990) and brain tumors (Ord and Thompson, 1952; Razon *et al.*, 1984). Interestingly, human oocytes were shown to express BuChE mRNA in a developmentally regulated fashion (Soreq *et al.*, 1987), although the enzyme activity that was evident in ovary homogenates was almost entirely AChE (Malinger *et al.*, 1989). Cholinergic signaling has also been implicated in chorionic villi, which express the BCHE gene (Zakut *et al.*, 1991) and in sperm motility, as suggested by the presence of ACh, AChE and ChAT (Bishop *et al.*, 1975, 1976) as well as BuChE in the mammalian sperm. Transgenic mice carrying the human BCHE gene displayed testicular amplification of this transgene and reduced fertility (Beeri *et al.*, 1994). Further, 1 μM of the ChAT inhibitor, 2-benzoyl methyl trimethyl ammonium, depressed sperm motility by 95% (Rama Sastry *et al.*, 1981), while ACh may either augment or impede sperm mobility in correlation with other characteristics of individual spermatozoa (Sanyal and Khanna, 1971; Beeri *et al.*, 1994).

Reports of peptidase activity accompanying purified AChE or BuChE have, in most cases been shown to be due to co-purification with other proteins. However, Balasubramanian and Bhanumathy (1993) report evidence of an intrinsic peptidase activity. This activity co-precipitates with an monoclonal antibody directed against BuChE. They point out that the tetrapeptide sequence, HXXE, separated by over 100 residues from another histidine residue, associated with Zn metallopeptidases, appears in human ChEs as $HGYE(X)_{107}H$ (BuChE residues 438-548 and AChE residues 440-550).

The effect of AChE, or the lack of the protein, in the hematopoietic system is difficult to reconcile with our understanding of AChE as the terminator of ACh action, as is the presence of AChE in the erythrocyte membrane. The sequence homology of AChE with the *Drosophila* adhesion proteins, glutactin and neurotactin had also been noted (De La Escalera *et al.*, 1990; Cygler *et al.*, 1993). This homology had been attributed to the evolutionary diversion of a protein featuring the α/β-hydrolase into contemporary serine α/β-hydrolases and other proteins without catalytic function, such as neurotactin and thyroglobulin (Taylor and Radic, 1994). A recent report (Layer *et al.*, 1993) on the G4 form of AChE in chick embryo tectal cell cultures, however, finds a direct quantitative relationship between inhibition of neurite growth and amount of an added anti-G4 antibody. This effect on neurite growth was not affected by at least one anti-ChE agent, echothiophate. Parallel morphological studies on retinal explants indicated that BW 284C51, which inhibits both AChE catalysis and neurite growth, visibly disrupted cell adhesion (Layer *et al.*, 1993). We note, also, that like

AChE, notably the erythrocyte membrane form, other GPI-anchored proteins have been proposed to have roles in cell-cell-signalling (Turner, 1994). Also, there has been a recent report of glioma cells in culture that when microinjected or transfected with DNA constructs expressing human AChE, showed dramatic morphological changes and rapid process extension (Karpel *et al.*, 1994b). These reports not only open a new arena of ChE studies, but breach the barrier to consideration of other non-enzymatic roles of ChEs, for instance in hematopoiesis, and may in time lead us to an understanding of the roles of several alternatively-spliced forms of AChE.

A recent study supports the idea of a developmental role for AChE. The destiny of hematopoietic stem cells was altered by the addition of antisense phosphorothioate oligonucleotides in a manner that indicated that AChE normally diverts stem cells to apoptosis (Soreq *et al.*, 1994). The blockage of this effect, by OP agents, for instance, may be the basis of the uncontrolled proliferation of the stem cells seen in leukemia (Brown *et al.*, 1990). A developmental role has also been suggested for BuChE and AChE in *ex vivo* developing chick motor axons based on the use of selective inhibitors (Layer, 1991; Layer *et al.*, 1988a,b, 1993). Thus, there is accumulating evidence for a developmental role (or roles) for ChEs which is not obviously related to their catalytic activity. Further experiments will be needed to determine whether this function is related to the presumed cell adhesion properties of these enzymes.

7. Human cholinesterase variants predict a genetic predisposition to differential responses to cholinergic drugs

Over several decades, large-scale population surveys of BuChE phenotypes were carried out. Tens of thousands of individuals have been screened from different continents and ethnic origins (Whittaker, 1986). Since the BCHE gene was cloned in 1986, more than twenty different naturally occurring mutations have been documented (Table 1), with the great majority of the variant phenotype individuals carrying the D70G substitution. These mutated proteins result in a variety of phenotypes, including the complete absence of any BuChE protein due to premature termination of protein synthesis (the "silent" mutations of Table 1). In at least one area of the brain, BuChE has been demonstrated in cells other than those that have AChE, suggesting a unique function (Graybiel *et al.*, 1981, 1982). It would be interesting to know if the distribution of AChE among brain cells is different in individuals with silent BuChE. The mere existence of a "silent" phenotype, where individuals do well in spite of having no BuChE activity, has been used to argue that BuChE has no important function. However, natural selection operates on the level of species, not individuals; what may be tolerated in an isolated individual may, over time and numbers, be disadvantageous to a community. Also, "knock-out" experiments have sometimes found no phenotypes for damaged genes; it would be reckless to conclude therefore, that all such genes have no important biological role. With the exception of two polymorphisms at the 5' and 3' non-translated sequence, no mutation in BCHE cDNA has yet been found that causes no alteration in the protein sequence. Together, the catalytically silent mutations comprise 0.001% of homozygotes, which is far less than the catalytically active variants (Whittaker, 1986). Even in the absence of a well-understood physiological role for BuChE, this in itself suggests a selection advantage for carriers of various genes coding for active proteins, as compared with "silent" gene carriers.

Interestingly, the largest and main coding exon, E2, has 15 of the known mutations found on the BCHE gene. Thus, the average incidence of mutability in the coding domain (approximately 1:100 nucleotides) is exceedingly high. The different BCHE variants were in most cases identified by the analysis of

sequences originating from individuals expressing a variant phenotype and not by a random screening of the population. Several of the variants (e.g. D70G) were simultaneously discovered in two continents, while many others were detected only once, an "orphan" allele.

One major physiological role of BuChE is thought to be as a scavenger of anti-ChE agents, thus protecting from inactivation the AChE of neuromuscular junctions and other cholinergic sites (Neville *et al.*, 1990a,b). This is deduced from the fact that BuChE interacts with a wider range of anti-ChE agents (Soreq *et al.*, 1992) and in certain cases (e.g. DFP and many carbamates) the rate of inactivation is considerably faster than that of AChE (Loewenstein *et al.*, 1994; Schwarz *et al.*, 1994). Accordingly, there must be an evolutionary pressure that accounts for the need for a scavenger of anti-ChE agents. There are many natural ChE inhibitors in the environment, including glycoalkaloids present in solanaceous plants (Gnatt *et al.*, 1994), fungal antibiotics like puromycin (Hersh, 1981) and its analogs, cocaine derivatives (Gatley, 1991), poisons from several species, like oysters (Abramson *et al.*, 1989), OPs from cyanobacteria (Carmichael, 1994), and polypeptides from snakes (fasciculin, Karlsson *et al.*, 1985) that are offensive or defensive weapon systems, metals (aluminum, scandium and yttrium, Marquis and Lerrick, 1982), and the carbamate of calabar beans, physostigmine (Taylor, 1990). Some of the above these ChE inhibitors are extremely poisonous. Several snake venoms contain peptides of 51-59 amino acid residues (e.g. fasciculin) that bind to AChE with K_d values as low as 10^{-10} M (Cervenansky *et al.*, 1990; Marchot *et al.*, 1993). However, it is perhaps significant that it is only the glycoalkaloids of the solanaceous food plants (tomato, potato, eggplant) that are inhibitors of both AChE and BuChE. Also, the uneven natural geographic distribution of these food plants must be seen alongside the large series of naturally occurring BuChE variants, also unevenly distributed among different populations -- the "atypical" BuChE mutation, D70G (heterozygote frequency <5% among Europeans and Americans and up to 11% of other groups; Whittaker, 1986; Ehrlich *et al.*, 1994) -- with variable affinities for them. Of all the classes of natural inhibitors of the ChEs, it seems that only for the glycoalkaloids may BuChE be imagined to have adapted as a scavanger.

The "atypical" mutation also confers resistance to inhibitors of pharmacological interest. It is clinically characterized by the inability of the affected enzyme to hydrolyze succinyl choline and dibucaine, and, compared to the wild-type BuChE, displays a specific activity of 25% of the wild-type enzyme, and at least 10-fold higher IC_{50} and K_i values for bambuterol, physostigmine and echothiophate. The affinity toward ACh is drastically reduced, although the K_m for BTCh is unchanged (Neville *et al.*, 1990a,b). If the mutant BuChE cannot scavenge anti-ChEs and reduce their serum levels, it will not protect synaptic AChE from their effects. The genetic variability of BuChE may well be the basis of the observed variability in the extent and intensity of responses to anti-ChE drugs. In fact, BuChE is reported to hydrolyze heroin (Fig. 4), which has a 4-fold higher K_m for the "atypical" variant than for the usual enzyme (Lockridge *et al.*, 1980). Clearly this has the potential for explaining variations in responses to this narcotic. As noted above, BuChE hydrolyzes the methyl ester bond of cocaine and its derivatives. The local anesthetic, procaine is hydrolyzed by BuChE, but it has a 15-fold higher K_m with the atypical variant than with the usual enzyme. Carriers of the atypical allele may not react substantially differently from carriers of the usual enzyme when receiving procaine i.m. as it would be exposed only minimally to BuChE. However, aspirin has a nearly 4-fold higher K_m with "atypical" BuChE (Valentino *et al.*, 1981). It acts after entering the blood stream where it is exposed to BuChE. This illustrates a potential for significant variations in response to pharmacological agents, arising from

natural variations in this drug-processing enzyme. Just how variant BuChEs may function in detoxifying cocaine and its derivatives is an important issue that simply has not yet been addressed. Furthermore, it was found that the oxime, 2-PAM, reactivates DFP-inactivated D70G BuChE at a 5-fold lower rate than does wild type BuChE. Variants having this mutation in tandem with one or two additional natural mutations (Y114H and S425P) display a rate of reactivation as much as 40-fold lower. Thus, the well established therapy of OP-intoxicated patients with 2-PAM, intended to regenerate active ChE, may well be less efficient in the case of carriers of variant BuChE (Schwarz *et al.*, 1994). Indeed, it has been reported that the response of OP-poisoned patients to 2-PAM therapy varies widely (Willems *et al.*, 1993).

The most frequent variant, "atypical" BuChE, was compared to the common human BuChE and AChE in its inhibition rate with several anti-ChEs of pharmacological interest (Loewenstein *et al.*, 1994). With common BuChE, the carbamates physostigmine, heptyl-physostigmine (Modulanum®) and SDZ ENA 713 had inactivation rates higher than or equal to AChE, but "atypical" BuChE had considerably lower rates. This suggests that BuChE usually reacts with the drugs in preference to AChE. However, heterozygous, and especially homozygous individuals carrying the "atypical" gene may well show increased sensitivity to the drugs. Moreover, the reversible inhibitor, THA, had a 300-fold higher IC_{50} value with "atypical" than with common BuChE. These findings may help explain the variations in response to anti-cholinesterase therapy that have been noted.

In contrast to the multitude of point mutations on the BCHE gene, only one single-point mutation has been observed in the ACHE gene. Its identity was discovered in several steps. The human ACHE gene was first demonstrated to present two co-dominant alleles at a single genetic locus (Coates and Simpson, 1972). Two decades later, replacement of His322 with asparagine (Lockridge *et al.*, 1992) was reported to be a common polymorphism. The substituted amino-acid residue is positioned at the surface of the AChE protein, apparently modifying one or more immunogenic epitopes in erythrocyte membrane AChE. Thus, this allele was identified as causative of the uncommon Yt^b blood (Bartels *et al.*, 1993) group, for which an incidence of 5% has been determined in the Caucasian population (Lewis *et al.*, 1987), and a considerably higher incidence in Israel (Levene *et al.*, 1987), recently confirmed by molecular genetic means (Ehrlich *et al.*, 1994).

There being two co-dominant alleles of the ACHE gene, creates a situation where homozygotes with either of these alleles will recognize the product of the other allele as a non-self antigen, very much analogous to the Rh blood group phenomenon. Therefore, Yt^b homozygotes may develop an immunological response to transfused blood from either hetero- or homozygotes of the common Yt^a phenotype. The result is hemolysis of the foreign erythrocytes, a condition that was well recognized before its molecular basis was discovered.

The plethora of mutations in the BCHE gene may be taken as further support for the idea that the major role of BuChE is to function as a scavenger, as the selection of proteins with modified properties will confer better resistance to specific cholinergic poisons. It may also be that the role of scavenger can conflict with another role for BuChE, for instance as a cell membrane element involved in development. In that case, a decreased affinity for an inhibitor in the environment may confer a selection advantage (Ehrlich *et al.*, 1994).

The very infrequent mutability of the ACHE gene, and the complete absence of any mutant with impaired function, had been taken as evidence that AChE is just too

sensitive an enzyme to tolerate any mutation. However, several developments call into question this interpretation. Although there are no known natural mutations that affect the catalytic function of AChE, there are mutations in the enzymatic system that forms the G4 form by addition of a non-catalytic tail to the enzyme (Hutchinson *et al.*, 1993). This results in a deficiency of AChE in motor endplates, with a consequent loss of motor function. It also raises the question of why there are no mutations of AChE that affect its function: if defective processing of the G4 form is compatible with life, even if with diminished motor function, are there no other possible AChE mutations that would preserve catalytic function? In fact, there are laboratory-produced site-directed mutations that have almost complete catalytic function. That similar natural mutations are not observed, may suggests that the gene is unusually protected from mutation, perhaps by binding proteins, or that the selection pressures against establishment of mutants in the population are based on features besides catalysis.

In conclusion, the biological roles of human ChEs and their multiple variants are far from being fully understood. Rather, the era of genetic engineering has provided novel tools with which to explore these as yet unknown functions and their implications for the field of therapeutics, an exciting endeavor.

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References

Abramson, S.N., Radic, Z., Manker, D., Faulkner, D.J. and Taylor, P. (1989) Onchidal: a naturally occurring irreversible inhibitor of acetylcholinesterase with a novel mechanism of action. *Mol. Pharmacol.* 36:349-354.

Ajmar, F., Garre, C., Sessarego, M., Ravazzolo, R., Barresi, R., Bianchi Searra, G. and Lituania, M. (1983) Expression of erythroid acetylcholinesterase in the K-562 leukemia cell line. *Cancer Res.* 43:5560-5563.

Aldridge, W.N. (1975) Survey of major points of interest about reactions of cholinesterases. *Croatia Chim. Acta* 47:225-233.

Aldridge, W.N. and Reiner, E. (1972) *Enzyme Inhibitors as Substrates. Interactions of Esterases with Esters of Organophosphorus and Carbamic Acids*. North-Holland Publishing Company, Amsterdam.

Andres, C., El Mourabit, M., Stutz, C., Mark, J. and Waksman, A. (1990) Are soluble and membrane-bound rat brain acetylcholinesterase different? *Neurochem. Res.* 15:1065-1072.

Andres, C., El Mourabit, M., Mark, J. and Waksman, A. (1992) A unique hydrophobic domain of rat brain globular acetylcholinesterase for binding to cell membranes. *Neurochem. Res.* 17:1247-1253.

Andres, C. Beeri, R., Lev-Lehman, E., Timberg, R., Shani, M. and Soreq, H. (1994) Transgenic overexpression of human acetylcholinesterase in mouse brain. *J. Neurochem.* 62 (Suppl. 1):S32A.

Anglade, P. (1987). Ultrastructural study of acetylcholinesterase activity in the intrapancreatic ganglia of the rat. *Cell. mol. Biol.* 33:63-67.

Arpagaus, M., Kott, M., Vatsis, K.P., Bartels, C.F. and La Du, B.N. (1990) Structure of the gene for human butyrylcholinesterase: evidence for a single copy; *Biochemistry* 29:124-131.

Ashani, Y., Shapira, S., Levy, D., Wolfe, A.D., Doctor, B.P. and Raveh, L. (1991) Butyrylcholinesterase and acetylcholinesterase prophylaxis against soman poisoning in mice. *Biochem. Pharmacol.* 41:37-41.

Atack, J.R., Perry, E.K., Bonham, J.R., Perry, R.H., Tomlinson, B.E., Blessed, G. and Fairbairn, A. (1983). Molecular forms of acetylcholinesterase in senile dementia of Alzheimer type: selective loss of the intermediate (10S) form. *Neurosci. Lett.* 40:199-204.

Atack, J.R., Perry, E.K., Bonham, J.R., Candy, J.M. and Perry, R.H. (1986) Molecular forms of acetylcholinesterase and butyrylcholinesterase in the aged human central nervous system. *J. Neurochem.* 47:263-277.

Augustinsson, K.B. (1948) Acetylcholinesterase: a study in comparative enzymology. *Acta physiol. Scand.* 15 (Suppl. 52):1-182.

Balasubramanian, A.S. and Bhanumathy, C.D. (1993) Noncholinergic functions of cholinesterases. *FASEB J.* 7:1354-1358.

Baldessarini, R.J. (1990) Drugs and the treatment of psychiatric disorders. In: *Pharmacological Basis of Therapeutics*, pp. 383-435, Gilman, A.G., Rall, T.W., Nies, A.S. and Taylor, P. (eds) Pergamon Press, New York.

Barak, D., Kronman, C., Ordentlich, A., Ariel, N., Bromberg, A., Marcus, D., Lazar, A., Velan, B., and Shafferman, A. (1994) Acetylcholinesterase peripheral anionic site degeneracy conferred by amino acid arrays sharing a common core. *J. biol. Chem.* 264:6296-6305.

Bartels, C.F., James, K. and La Du, B.N. (1992a) DNA mutations associated with the human butyrylcholinesterase J-variant. *Am. J. hum. Genet.* 50:1104-1114.

Bartels, C.F., Jensen, F.S., Lockridge, O., van der Spek, A.F.L., Rubenstein, H.M. Lubrano, T. and La Du, B.N. (1992b) DNA mutation associated with the human butyrylcholinesterase K-variant and its linkage to the atypical variant mutation and other polymorphic sites. *Am. J. hum. Genet.* 50:1086-1103.

Bartels, C.F., Zelinski, T. and Lockridge, O. (1993) Mutation at codon 322 in the human acetylcholinesterase (ACHE) gene accounts for YT blood group polymorphism. *Am. J. hum Genet.* 52:926-936.

Beeri, R., Gnatt, A., Lapidot-Lifson, Y., Ginzberg, D., Shani, M., Soreq, H. and Zakut, H. (1994). Testicular amplification and impaired transmission of human butyrylcholinesterase cDNA in transgenic mice. *Hum. Reprod.* 9:284-292.

Ben Aziz-Aloya, R., Seidman, S., Timberg, R., Sternfeld, M., Zakut H. and Soreq, H. (1993) Expression of a human acetylcholinesterase promoter-reporter construct in developing neuromuscular junctions of *Xenopus* embryos. *Proc. natl. Acad. Sci. U.S.A.* 90:2471-2475.

Bianchi Searra, G.L., Garre, C. Ravazzolo, R., Coviello, D. and Origoni, P. (1986) Coordinated expression of acetylcholinesterase and hemoglobin in K562 cells induced to terminal differentiation by cytosine arabinoside (ara-C). *Cell. Biol. Int. Report.* 10:167.

Bishop, M.R., Rama Sastry, B.V., Schmidt, D.E., and Harbison, R.D. (1975). Spermic cholinergic system and occurrence of acetylcholine and other quaternary ammonium compounds in mammalian spermatozoa. *Toxicol. appl. Pharmacol.* 33:733-734.

Bishop, M.R., Rama Sastry, B.V., Schmidt, D.E., and Harbison, R.D. (1976). Occurrence of choline acetyltransferase and acetylcholine and other quaternary ammonium compounds in mammalian spermatozoa. *Biochem. Pharmacol.* 25:1617-1622.

Brown, L.M., Blair, A., Gibson, R., Everett, G.D., Cantor, K.P., Schuman, L.M., Burmeister, L.F., Van Lier, S.F. and Dick, F. (1990) Pesticide exposures and other agricultural risk factors for leukemia among men in Iowa and Minnesota. *Cancer Res.* 50:6585-6591.

Bucht, G., Artursson, E., Haggstrom, B., Osterman, A. and Hjalmarsson, K. (1992) Structurally important residues in the region ser91 to asn98 of *Torpedo* acetylcholinesterase. In: *Multidisciplinary Approaches to Cholinesterase Functions, Proceedings of the Thirty-Sixth Oholo Conference on Multidisciplinary Approaches to Cholinesterase Functions, Eilat, Israel, 6-10 April 1992*, pp. 185-188, Shafferman, A,, Velan, B. (eds) Plenum Press, New York.

Burstein, S.A., Adamson, J.W. and Harker, L.A. (1980). Megakaryocytopoiesis in culture: modulation by cholinergic mechanisms. *J. cell. Physiol.* 103:201-208.

Carmichael, W.W. (1994) The toxins of cyanobacteria. *Sci. Amer.* 270:64-72.

Cauet, G., Friboulet, A. and Thomas, D. (1987) Horse serum butyrylcholinesterase kinetics: a molecular mechanism based on inhibition studies with dansylaminoethyltrimethylammonium. *Biochem. cell. Biol.* 65:529-535.

Cervenansky, C., Dajas, F., Harvey, A.L. and Karlsson, E. (1990) The fasciculins. In: *Snake Toxins*, pp. 303-321, Harvey, A.L. (ed) Pergamon Press, New York.

Changeux, J.-P. (1966) Responses of acetylcholinesterase from *Torpedo marmorata* to salts and curarizing drugs. *Mol. Pharmacol.* 2:369-392.

Clement, J.G. (1991) Hypothermia: limited tolerance to repeated soman administration and cross-tolerance to oxotremorine. *Pharmacol. Biochem. Behav.* 39:305-312.

Coates, J.T. and Simpson, N.E. (1972). Genetic variation in human erythrocyte acetylcholinesterase. *Science* 175:1466-1477.

Coleman, B.A., Michel, L. and Oswald, R. (1987). Interaction of a benzomorphan opiate with acetylcholinesterase and the nicotinic acetylcholine receptor. *Mol. Pharmacol.* 32:456-462.

Coyle, J.T., Price, D.L. and DeLong, M.R. (1983) Alzheimer's disease: a disorder of cortical cholinergic innervation. *Science* 219:1184-1190.

Cygler, M., Schrag, J.D., Sussman, J.L., Harel, M., Silman, I. and Doctor, B.P. (1993). Relationship between sequence conservation and three-dimensional structure in a large family of esterases, lipases, and related proteins. *Protein Sci.* 2:366-382.

Davis, R.E., Emmerling, M.R., Jaen, J.C., Moos, W.H. and Spiegel, K. (1993) Therapeutic intervention in dementia. *Crit. Rev. Neurobiol.* 7:41-83.

De Kosky, S.T. and Scheff, S.W. (1990) Synapse loss in frontal cortex biopsies in Alzheimer's disease: correlation with cognitive severity. *Ann. Neurol.* 27:457-464.

De La Escalera, S., Bockamp, E.-O., Moya, F., Piovant, M. and Jimenez, F. (1990) Characterization and gene cloning of neurotactin, a *Drosophila* transmembrane protein related to cholinesterases. *EMBO J.* 9:3593-3601.

Doctor, B.P., Raveh, L., Wolfe, A.D., Maxwell, D.M., Ashani, Y. (1991) Enzymes as pretreatment drugs for organophosphate toxicity. *Neurosci. behav. Rev.* 15:123-128.

Doctor B.P., Blick, D.W., Caranto, G., Castro, C.A., Gentry, M.K., Maxwell, D.M., Murphy, M.R., Schultz, M., Waibel, K. and Wolfe, A.D. (1993) Cholinesterases as scavengers for organophosphorus compounds: protection of primate performance against soman toxicity. *Chemico-Biological Interactions* 87:285-294.

Dretchen, K.L., Singh, A., Bradley, R.M. and Lynch, T.J. (1992) Protection against cocaine toxicity by human butyrylcholinesterase (BCHE) in rats (abstract). *FASEB J.* 6:A1282.

Drews, U. (1975). *Cholinesterase in embryonic development, Progress in Histochemistry and Cytochemistry Vol.* 7, Fischer, Stuttgart.

Dreyfus, P.A., Seidman, S., Pincon-Raymond, M., Murawsky, M., Rieger, F., Schejter, E., Zakut, H. and Soreq, H. (1989). Tissue-specific processing and polarized compartmentalization of clone-produced cholinesterase in microinjected *Xenopus* oocytes. *Cell. molec. Neurobiol.* 9:323-341.

Eckerson, H.W., Oseroff, A., Lockridge, O., and La Du, B.N. (1983) Immunological comparison of the usual and atypical human serum cholinesterase phenotypes. *Biochem. Genet.* 21:93-108.

Ehrlich, G., Viegas-Pequignot, E., Ginzberg, D., Sindel, L., Soreq, H. and Zakut, H. (1992). Mapping the human acetylcholinesterase gene to chromosome 7q22 by fluorescent in situ hybridization coupled with selective PCR amplification from a somatic hybrid cell panel and chromosome-sorted DNA libraries. *Genomics* 13:1192-1197.

Ehrlich, G., Ginzberg, D., Loewenstein, Y., Glick, D., Kerem, B., Ben-Ari, S., Zakut, H. and Soreq, H. (1994) Population diversity and distinct haplotype frequencies associated with ACHE and BCHE genes of Israeli Jews from trans-Caucasian Georgia and from Europe. *Genomics* 21 (in press).

Enz, A., Boddeke, H., Gray, J. and Spiegel, R. (1991). Pharmacologic and clinicopharmacologic properties of SDZ ENA 713, a centrally selective acetylcholinesterase inhibitor. *Ann. N.Y. Acad. Sci.*

Enz, A., Amstutz, R., Boddeke, H. Gmelin, G. and Malanowski, J. (1993) Brain selective inhibition of acetylcholinesterase: a novel approach to therapy for Alzheimer's disease. *Prog. Brain Res.* 98:431-437

Erickson, J.D., Varoqui, H., Schafer, M.K.-H., Modi, W., Diebler, M.-F., Weihe, E., Rand, J., Eiden, L.E., Bonner, T.I. and Usdin, T.B. Functional identification of a vesicular acetylcholine transporter and its expression from a "cholinergic" gene locus. *J. biol. Chem.* (in press).

Fischer, M., Ittah, A., Liefer, I. and Gorecki, M. (1993). Expression and reconstitution of biologically active human acetylcholinesterase from Escherichia coli. *Cell. mol. Neurobiol.* 13:25-38.

Fishman, E.B., Siek, G.C., MacCallum, R.D., Bird, E.D., Volicer, L. and Marquis, J.K. (1986). Distribution of the molecular forms of acetylcholinesterase in human brain: alterations in dementia of the Alzheimer type. *Ann. Neurol.* 19:246-252.

Fournier, D., Mutero, A., Pralavorio, M. and Bride, J.M. (1992) *Drosophila* acetylcholinesterase: analysis of structure and sensitivity to insecticides by *in vitro* mutagenesis and expression. In: *Multidisciplinary Approaches to Cholinesterase Functions, Proceedings of the Thirty-Sixth Oholo Conference on Multidisciplinary Approaches to Cholinesterase Functions, Eilat, Israel, 6-10 April 1992*, pp. 75-81, Shafferman, A,, Velan, B. (eds) Plenum Press, New York.

Foutz, A.S., Boudinot, E. and Denavit-Saubie, M. (1987). Central respiratory depression induced by acetylcholinesterase inhibition: involvement of anaesthesia. *Eur. J. Pharmacol.* 142:207-213.

Fuentes, M.E., Rosenberry, T.L. and Inestrosa, N.C. (1988). A 13 kDa fragment is responsible for the hydrophobic aggregation of brain G4 acetylcholinesterase. *Biochem. J.* 256:1047-1050.

Futerman, A.H., Barton, P.L., Fiorini, R.M., Low, M.G., Sherman, W.R. and Silman, I. (1985a). The involvement of phosphatidylinositol in the anchoring of hydrophobic forms of acetylcholinesterase to the plasma membrane. In: *Molecular Basis of Nerve Activity, Proceedings of the International Symposium in Memory of David Nachmansohn (1899-1983), Berlin, (West) Germany, 11-13 October 1984*, pp. 635-650, Changeux, J.-P., Hucho, F., Maelicke, A., and Neumann, E. (eds) Walter de Gruyter, Berlin.

Futerman, A.H., Low, M.G., Michaelson, D.M. and Silman, I. (1985b). Solubilization of membrane-bound acetylcholinesterase by a phosphatidylinositol-specific phospholipase C. *J. Neurochem.* 45:1487-1494.

Gatley, S.J. (1991) Activities of the enantiomers of cocaine and some related compounds as substrates and inhibitors of plasma butyrylcholinesterase. *Biochem. Pharmacol.* 41:1249-1254.

Getman, D.K., Eubanks, J.H., Camp, S., Evans, G.A. and Taylor, P. (1992) The human gene encoding acetylcholinesterase is located on the long arm of chromosome 7. *Am. J. hum. Genet.* 51:170-177.

Gibney, G., Camp, S., Dionne, M., MacPhee-Quigley, K. and Taylor, P. (1990). Mutagenesis of essential functional residues in acetylcholinesterase. *Proc. natl. Acad. Sci. U.S.A.* 87:7546-7550.

Gilson, M.K., Straatsma, T.P., McCammon, J.A., Ripoll, D.R., Faerman, C.H., Axelsen, P.H., Silman, I. and Sussman, J.L. (1994) Open "back door" in a molecular dynamics simulation of acetylcholine. *Science* 263:1276-1278.

Gnatt, A., Prody, C.A., Zamir, R., Lieman-Hurwitz, J., Zakut, H. and Soreq, H. (1990). Expression of alternatively terminated unusual human butyrylcholinesterase messenger RNA transcripts, mapping to chromosome 3q26-ter, in nervous system tumors. *Cancer Res.* 50:1983-1987.

Gnatt, A., Ginzberg, D., Lieman-Hurwitz, J., Zamir, R., Zakut, H. and Soreq, H. (1991). Human acetylcholinesterase and butyrylcholinesterase

are encoded by two distinct genes. *Cell. mol. Neurobiol.* 11:91-104.

Gnatt, A., Loewenstein, Y. and Soreq, H. (1992) Molecular dissection of functional domains in human cholinesterases expressed in microinjected Xenopus oocytes. In: *Multidisciplinary Approaches to Cholinesterase Functions, Proceedings of the Thirty-Sixth Oholo Conference on Multidisciplinary Approaches to Cholinesterase Functions, Eilat, Israel, 6-10 April 1992*, pp. 157-164, Shafferman, A,, Velan, B. (eds) Plenum Press, New York.

Gnatt, A., Loewenstein, Y., Yaron, A., Schwarz, M. and Soreq, H. (1994) Site-directed mutagenesis of active site residues reveals plasticity of human butyrylcholinesterase in substrate and inhibitor interactions. *J. Neurochem.* 62:749-755.

Goonetilleke, A., de Belleroche, J. and Guiloff, R.J. (1994) Motor neurone disease. *Essays Biochem.* 28:27-45.

Graybiel, A.M., Pickel, V.M., Joh, T.H., Reis, D.J. and Ragsdale, C.W., Jr. (1981). Direct demonstration of a correspondence between the dopamine islands and acetylcholinesterase patches in the developing striatum. *Proc. natl. Acad. Sci. U.S.A.* 78:5871-5875.

Graybiel, A.M. and Ragsdale, C.W., Jr. (1982). Pseudocholinesterase staining in the primary visual pathway of the macaque monkey. *Nature* 299:439-442.

Greer, J. (1990) Comparative modeling methods: application to the family of the mammalian seine proteases. *Proteins Struct. Funct. Genet.* 7:317-334.

Gregor, V.E., Emmerling, M.R., Lee, C. and Moore, C.J. (1992) The synthesis and *in vitro* acetylcholinesterase and butyrylcholinesterase inhibitory activity of tacrine (Cognex®) derivatives. *Bioorgan. med. Chem. Lett.* 8:861-864.

Gurney, M.E., Pu, H., Chiu, A.Y., Dal Canto, M.C., Polchow, C.Y., Alexander, D.D., Caliendo, J., Hentati, A., Kwon, Y.W., Deng, H.-X., Chen, W., Zhai, P., Sufit, R.L. and Siddique, T. (1994) Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. *Science* 264:1772-1775.

Hall, L.M. and Malcolm C.A. (1991) The acetylcholinesterase gene of Anopheles stephensi. *Cell. mol. Neurobiol.* 11:131-141.

Hall, Z.W. (1973). Multiple forms of acetylcholinesterase and their distribution in endplate and non-endplate regions of rat diaphragm muscle. *J. Neurobiol.* 4:343-361.

Harel, M., Sussman, J.L., Krejci, E., Bon, S., Chanal, P., Massoulie, J. and Silman, I. (1992). Conversion of acetylcholinesterase to butyrylcholinesterase: modeling and mutagenesis. *Proc. natl. Acad. Sci. U.S.A.* 89:10827-10831.

Harel, M., Schalk, I., Ehret-Sabatier, L., Bouet, F., Goeldner, M., Hirth, C., Axelsen, P.H., Silman, I. and Sussman, J.L. (1993) Quaternary ligand binding to aromatic residues in the active-site gorge of act acetylcholinesterase. *Proc. natl. Acad. Sci. U.S.A.* 90:9031-9035.

Hersh, L.B. (1981). Inhibition of aminopeptidase and acetylcholinesterase by puromycin and puromycin analogs. *J. Neurochem.* 36:1594-1596.

Hokin, M.R. and Hokin, L.E. (1953) Enzyme secretion and the incorporation of P^{32} into phospholipids of pancreas slices. *J. biol. Chem.* 203:967-977.

Hutchinson, D.O., Walls, T.J., Nakano, S., Camp, S., Taylor, P., Harper, C.M., Groover, R.V., Peterson, H.A., Jamieson, D.G. and Engel, A.G. (1993) Congenital endplate acetylcholinesterase deficiency. *Brain* 116:633-653.

Inestrosa, N.C., Roberts, W.L., Marshall, T.L. and Rosenberry, T.L.

(1987). Acetylcholinesterase from bovine caudate nucleus is attached to membranes by a novel subunit distinct from those of acetylcholinesterases in other tissues. *J. biol. Chem.* 262:4441-4444.

Isenschmid, D.S., Levine, B.S. and Caplan, Y.H. (1989) A comprehensive study of the stability of cocaine and its metabolites. *J. anal. Toxicol.* 13:250-256.

Kambam, J.R., Naukam, R. and Berman, M.L. (1992) Inhibition of pseudocholinesterase activity protects from cocaine-induced cardiorespiratory toxicity in rats. *J. lab. clin. Med.* 119:553-556.

Kambam, J., Mets, B., Hickman, R.M., Janicki, P., James, M.F. and Kirsch, R.E. (1993) The effects of inhibition of plasma cholinesterase and hepatic microsomal enzyme activity on cocaine, benzoylecgonine, ecgonine methyl ester, and norcocaine blood levels in pigs. *J. lab. clin. Med.* 120:323-328.

Karlsson, E., Mbugua, P.M. and Rodriguez-Ithurralde, D. (1985) Anticholinesterase toxins. *Pharmacol. Ther.* 30:259-276.

Karnovsky, M.J. and Roots, L. (1964). A "direct coloring" thiocholine method for cholinesterases. *J. Histochem. Cytochem.* 12:219-221.

Karpel, R., Ben Aziz-Aloya, R., Sternfeld, M., Ehrlich, G., Ginzberg, D., Tarroni, P., Clementi, F., Zakut, H. and Soreq, H. (1994a) Expression of three alternative acetylcholinesterase messenger RNAs in human tumor cell lines of different tissue origins. *Exp. Cell Res.* 210:268-277.

Karpel, R., Sternfeld, M., Ginzberg, D., Guhl, E., Graessmann, A. and Soreq, H. (1994b) Overexpression of acetylcholinesterase variants induces morphogenic changes in rat glioma cells. *J. Neurochem.* 63 (Suppl. 1):S63D.

Knapp, M.J., Knopman, D.S., Solomon, P.R., Pendlebury, W.W., David, C.S., Gracon, S.I. (1994) A 30-week randomized controlled trial of high-dose tacrine in patients with Alzheimer's disease. *J. Am. med. Assn.* 271:985-991.

Kostovic, I. and Goldman-Rakic, P.S. (1983). Transient cholinesterase staining in the mediodorsal nucleus of the thalamus and its connections in the developing human and monkey brain. *J. comp. Neurol.* 219:431-447.

Kristt, D.A. (1983). Acetylcholinesterase in the ventrobasal thalamus: transience and patterning during ontogenesis. *Neuroscience* 10:923-939.

Kristt, D.A. and Kasper, E.K. (1983). High density of cholinergic-muscarinic receptors accompanies high-intensity of acetylcholinesterase staining in layer-IV of infant rat somatosensory cortex. *Dev. Brain Res.* 8:373-376.

Lapidot-Lifson, Y., Prody, C.A., Ginzberg, D., Meytes, D., Zakut, H. and Soreq, H. (1989) Coamplification of human acetylcholinesterase and butyrylcholinesterase genes in blood cells: correlation with various leukemias and abnormal megakaryocytopoiesis. *Proc. natl. Acad. Sci. U.S.A.* 86:4715-4717.

Landwehrmeyer, B., Probst, A., Palacios, J.M. and Mengod, G. Expression of acetylcholinesterase messenger RNA in human brain: an *in situ* hybridization study. *Neuroscience* 57:615-634.

Layer, P.G. (1991). Cholinesterases during development of the avian nervous system. *Cell. mole. Neurobiol.* 11:7-33.

Layer, P.G., Alber, R. and Rathjen, F.G. (1988a). Sequential activation of butyrylcholinesterase in rostral half somites and acetylcholinesterase in motoneurons and myotomes preceding growth of motor axons. *Development* 102:387-396.

Layer, P.G., Rommel, S., Bulthoff, H. and Hengstenberg, R. (1988b). Independent spatial waves of biochemical differentiation along the surface of chicken brain as revealed by the sequential expression of acetylcholinesterase. *Cell Tissue Res.* 251:587-595.

Layer, P.G., Weikert, T., Alber, R. (1993) Cholinesterases regulate neurite growth of chick nerve cells in vitro by means of a non-enzymatic mechanism. *Cell Tissue Res.* 273:219-226.

Levene, C., Bar-Shany, S., Manny, N., Moulds, J.J. and Cohen, T. (1987) The Yt blood groups in Israeli Jews, Arabs, and Druse. *Transfusion* 27:471-474.

Lev-Lehman, E., Ginzberg, D., Hornreich, G., Ehrlich, G., Meshorer, A., Eckstein, A., Soreq, H. and Zakut, H. (1994) Antisense inhibition of acetylcholinesterase gene expression causes transient hematopoietic alterations *in vivo*. *Gene Therapy* 1:127-135.

Lewis, M., Kaita, H., Philipps, S., McAlpine, P.J., Wong. P., Giblett, E.R. and Anderson, J. (1987) The Yt blood group system (ISBT No.011). *Vox Sang.* 53:52-56.

Lockridge, O. (1990). Genetic variants of human serum cholinesterase influence metabolism of the muscle relaxant succinylcholine. *Pharmacol. Ther.* 47:35-60.

Lockridge, O., Mottershaw-Jackson, N., Eckerson, H.W., La Du, B.N. (1980) Hydrolysis of diacetylmorphine (heroin) by human serum cholinesterase. *J. Pharmac. exp. Ther.* 215:1-8.

Lockridge, O., Bartels, C.F., Vaughan, T.A., Wong, C.K., Norton, S.E. and Johnson, L. (1987). Complete amino acid sequence of human serum cholinesterase. *J. biol. Chem.* 262:549-557.

Lockridge, O., Bartels, C.F., Zelinski, T., Jbilo, O. and Kris, M. (1992) Part 1: Genetic variant of human acetylcholinesterase. Part 2: SV-40 transformed cell lines, for example COS-1, but not parental untransformed cell lines, express butyrylcholinesterase (BCHE). In: *Multidisciplinary Approaches to Cholinesterase Functions, Proceedings of the Thirty-Sixth Oholo Conference on Multidisciplinary Approaches to Cholinesterase Functions, Eilat, Israel, 6-10 April 1992*, pp. 53-59, Shafferman, A,, Velan, B. (eds) Plenum Press, New York.

Loewenstein, Y., Gnatt, A., Neville, L.F. and Soreq, H. (1993a) A chimeric human cholinesterase: identification of interaction sites responsible for sensitivity to acetyl- or butyrylcholinesterase-specific ligands. *J. mol. Biol.* 234:289-296.

Loewenstein, Y., Gnatt, A., Neville, L.F., Zakut, H. and Soreq, H. (1993b) Structure-function relationship studies in human cholinesterases reveal genomic origins for individual variations in cholinergic drug responses. *Prog. Neuro-Psychophamacol. biol. Psychiat.* 17:905-926.

Loewenstein, Y., Liao, J., Norgaard-Pedersen, B., Zakut, H. and Soreq, H. (1994) Faster inhibition rates of normal BuChE as compared with AChE and the D70G "atypical" BuChE mutant predict individual variabilities in response to anticholinesterase therapy. *J. Neurochem.* 63 (Suppl. 1):S6D.

Malinger, G., Zakut, H. and Soreq, H. (1989). Cholinoceptive properties of human primordial, preantral, and antral oocytes: In situ hybridization and biochemical evidence for expression of cholinesterase genes. *J. mol. Neurosci.* 1:77-84.

Marchot, P., Khelif, A., Ji, Y.-H., Mansuelle, P. and Bougis, P.E. (1993) Binding of ^{125}I-fasciculin to rat brain acetylcholinesterase: the complex still binds diisopropyl fluorophosphate. *J. biol. Chem.* 268:12458-12567.

Marquis, J.K. and Fishman, E.B. (1985). Presynaptic acetylcholinesterase. *Trends pharmacol. Sci.* 6:387-388.

Marquis, J.K. and Lerrick, A.J. (1982) Noncompetitive inhibition by aluminum, scandium, and yttrium of acetylcholinesterase from Electrophorus electricus. *Biochem. Pharmacol.* 31:1437-1440.

Marrs, T.C. (1993) Organophosphate poisoning. *Pharmac. Ther.* 58:51- 66.

Masson, P. (1991) Molecular heterogeneity of human plasma cholinesterase. In: *Cholinesterases, Structure, Function, Mechanism, Genetics, and Cell Biology, Proceedings of the Third International Meeting on Cholinesterases*, La Grande Motte, France, 12-16 May 1990, pp. 42-46, Massoulie, J., Bacou, F., Barnard, E., Chatonnet, A., Doctor, B.P. and Quinn, D.M. (eds) American Chemical Society, Washington.

Masson, P., Adkins, S., Gouet, P. and Lockridge, O. (1993) Recombinant human butyrylcholinesterase G390V, the fluoride-2 variant, expressed in Chinese hamster ovary cells, is a low affinity variant. *J. biol. Chem.* 268:14329-14341.

Massoulie, J. and Bon, S. (1982) The molecular forms of cholinesterase and acetylcholinesterase in vertebrates. *Annu. Rev. Neurosci.* 5:57-106.

Massoulie, J., Pezzementi, L., Bon, S., Krejci, E., Vallette, F.M. (1993) Molecular and cellular biology of the cholinesterases. *Prog. Neurobiol.* 41:31-91.

Maulet, Y., Camp, S., Gibney, G., Rachinsky, T.L., Ekstrom, T.J. and Taylor, P. (1990) Single gene encodes glycophospholipid-anchored and asymmetric acetylcholinesterase forms: alternative coding exons contain inverted repeat sequences. *Neuron* 4:289-301.

Maxwell, D.M., Castro, C.A., De La Hoz, D.M., Gentry, M.K., Gold, M.B., Solana, R.P., Wolfe, A.D. and Doctor, B.P. (1992) Protection of rhesus monkeys against soman and prevention of performance decrement by pretreatment with acetylcholinesterase. *Toxicol. appl. Pharmacol.* 115:44-49.

McGuire, M.C. Nogueira, C.P., Bartels, C.F., Lightstone, H., Hajra, A., van der Spek, A.F.L., Lockridge, O. and La Du, B.N. (1989) Identification of the structural mutation responsible for the dibucaine-resistant (atypical) variant form of human serum cholinesterase. *Proc. natl. Acad. Sci. U.S.A.* 86:953-957.

McMahan, U.J., Sanes, J.R. and Marshall, L.M. (1978) Cholinesterase is associated with the basal lamina at the neuromuscular junction. *Nature* 271:172-174.

McTiernan, C., Adkins, S., Chatonnet, A., Vaughan, T.A., Bartels, C.F., Kott, M., Rosenberry, T.L., La Du, B.N. and Lockridge, O. (1987) Brain cDNA clone for human cholinesterase. *Proc. natl. Acad. Sci. U.S.A.* 84:6682-6686.

Meflah, K., Bernard, S. and Massoulie, J. (1984) Interaction with lectins indicates differences in the carbohydrate composition of the membrane-enzymes acetylcholinesterase and 5'-nucleotidase in different cell types. *Biochemie* 66:59-69.

Minthon, L., Gustafson, L., Dalfelt, G., Hagberg, B., Nilsson, K., Risberg, J., Rosen, I., Seiving, B. and Wendt, P.E. (1993) Oral tetrahydroaminoacridine treatment of Alzheimer's disease evaluated clinically and by regional cerebral blood flow and EEG. *Dementia* 4:32-42, 1993.

Mullan, M. and Crawford, F. (1993) Genetic and molecular advances in Alzhiemer's disease. *Trends Neurosci.* 16:398-403.

Muratani, P., Hada, T., Yamamoto, Y., Kaneko, Y., Shigero, Y., Ohue, T., Furuyama, J. and Higashino, K. (1991) Inactivation of the cholinesterase gene by *Alu* insertion: possible mechanism for human gene transposition. *Proc. natl. Acad. Sci. U.S.A.* 88:11315-11319.

Mutero, A. and Fournier, D. (1992) Post-translational modifications of *Drosophila* acetylcholinesterase; in vitro expression in Xenopus oocytes. *J. biol. Chem.* 267:1695-1700.

Mutero, A., Pralavorio, M., Simeon, V. and Fournier, D. (1992) Catalytic properties of cholinesterases: importance of tyrosine 109 in *Drosophila* protein. *NeuroReport* 3:39-42.

Neville, L.F., Gnatt, A., Loewenstein, Y. and Soreq, H. (1990a) Aspartate-70 to glycine substitution confers resistance to naturally occurring and synthetic anionic-site ligands on in-ovo produced human butyrylcholinesterase. *J. Neurosci. Res.* 27:452-460.

Neville, L.F., Gnatt, A., Padan, R., Seidman, S. and Soreq, H. (1990b). Anionic site interactions in human butyrylcholinesterase disrupted by two single point mutations. *J. biol. Chem.* 265:20735-20738.

Neville, L.F., Gnatt, A., Loewenstein, Y., Seidman, S., Ehrlich, G. and Soreq, H. (1992) Intramolecular relationships in cholinesterase revealed by oocyte expression of site-directed and natural variants of human BCHE. *EMBO J.* 11:1641-1649.

Nogueira, C.P., McGuire, M.C., Graeser, C., Bartels, C.F., Arpagaus, M., Lightstone, H., Lockridge, O. and La Du, B.N. (1990). Identification of a frameshift mutation responsible for the silent phenotype of human serum cholinesterase, Gly 117(GGT→GGAG). *Am. J. hum. Genet.* 46:934-942.

Olianas, M.C., Onali, P., Schwartz, J.P., Neff, N.H. and Costa, E. (1984). The muscarinic receptor adenylate cyclase complex of rat striatum: desensitization following chronic inhibition of acetylcholinesterase activity. *J. Neurochem.* 42:1439-1443.

Ollis, D.L., Cheah, E., Cygler, M., Dijkstra, B., Frolow, F., Franken, S.M., Harel, M., Remington, S.J., Silman, I., Schrag, J.D., Sussman, J.L., Versschveren, K.H.G. and Goldman, A. (1992) The α/β hydrolase fold. *Prot. Eng.* 5:197-211.

Ord, M.G. and Thompson, R.H. (1952). Pseudocholinesterase activity in the central nervous system. *Biochem. J.* 51:245-251.

Ordentlich, A., Barak, D., Kronman, C., Flashner, Y., Leitner, M., Ariel, N., Cohen, S., Velan, B. and Shafferman, A. (1993a). Dissection of the human acetylcholinesterase active center determinants of substrate specificity. Identification of residues constituting the anionic site, the hydrophobic site, and the acyl pocket. *J. biol. Chem.* 268:17083-17095.

Ordentlich, A., Kronman, C., Barak, D., Stein, D., Ariel, N., Marcus, D., Velan, B., and Shafferman, A. (1993b) Engineering resistance to "aging" of phosphylated human acetylcholinesterase: role of hydrogen bond network in the active center. *FEBS Lett.* 334:215-220.

Ott, B.R. and Lannon, M.C. (1992) Exacerbation of parkinsonism by tacrine. *Clin. Neuropharmacol.* 15:322-325.

Patinkin, D., Seidman, S., Eckstein, F., Benseler, F., Zakut, H. and Soreq, H. (1990) Manipulations of cholinesterase gene expression modulate murine megakaryocytopoiesis in vitro. *Mol. cell. Biol.* 10:6046-6050.

Patinkin, D., Lev-Lehman, E., Zakut, H., Eckstein, F. and Soreq, H. Antisense inhibition of butyrylcholinesterase gene expression predicts adverse hematopoietic consequences to cholinergic inhibitors. *Cell. mol. Neurobiol.* (in press).

Percy, M.E., Markovic, V.D., Dalton, A.J., McLachlan, D.R.C, Berg, J.M. Rusk, A.C.M., Somerville, M.J., Chodakowski, B. and Andrews, D.F. (1993) Age-associated chromosome 21 loss in Down syndrome: possible relevance to mosaicism and Alzheimer disease. *Am. J. med. Genet.* 45:584-588.

Perry, E.K., Tomlinson, B.E., Blessed, G., Bergmann, K., Gibson, P.H. and Perry, R.H. (1978) Correlation of cholinergic abnormalities with senile plaques and mental test scores in senile dementia. *Br. med. J.* 2:1457-1459.

Primo-Parmo, S.L., Bartels, C.F., Lightstone, H., van der Spek, A.F.L., La Du, B.N. (1992) Heterogeneity of the silent phenotype of human butyrylcholinesterase - identification of eight new mutations. In: *Multidisciplinary Approaches to Cholinesterase Functions, Proceedings of the Thirty-Sixth Oholo Conference on Multidisciplinary Approaches to Cholinesterase Functions, Eilat, Israel, 6-10 April 1992*, pp. 61-64, Shafferman, A,, Velan, B. (eds) Plenum Press, New York.

Prody, C.A., Gnatt, A., Zevin-Sonkin, D., Gnatt, A., Goldberg, O. and Soreq, H. (1987). Isolation and characterization of full-length cDNA clones coding for cholinesterase from fetal human tissues. *Proc. natl. Acad. Sci. U.S.A.* 84:3555-3559.

Prody, C.A., Dreyfus, P., Zamir, R., Zakut, H. and Soreq, H. (1989). De novo amplification within a "silent" human cholinesterase gene in a family subjected to prolonged exposure to organophosphorous insecticides. *Proc. natl. Acad. Sci. U.S.A.* 86:690-694.

Radic, Z., Gibney, G., Kawamoto, S., MacPhee-Quigley, K., Bongiorno, C. and Taylor, P. (1992) Expression of recombinant acetylcholinesterase in a baculovirus system: kinetic properties of glutamate 199 mutants. *Biochemistry* 31:9760-9767.

Radic, Z., Duran, R., Vellom, D.C., Li, Y., Cervenansky, C. and Taylor, P. (1994) Site of fasciculin interactions with acetylcholinesterase. *J. biol. Chem.* 269:11233-11239.

Rakonczay, Z. and Brimijoin, S. (1988). Biochemistry and pathophysiology of the molecular forms of cholinesterases. In: *Subcellular Biochemistry, Vol. 12, Immunological Aspects*, pp. 335-378, Harris, J.R. (ed) Plenum Press, New York.

Rama Sastry, B.V., Janson, V.E. and Chaturverdi, A.K. (1981) Inhibition of human sperm motility by inhibitors of choline acetyltransferase. *J. Pharmacol. exp. Ther.* 216:378-384.

Ratner, D., Oren, B. and Vigder, K. (1983). Chronic dietary anticholinesterase poisoning. *Isr. J. med. Sci.* 19, 810-814.

Raveh, L., Ashani, Y., Levy, D., De La Hoz, D., Wolfe, A.D.and Doctor, B.P. (1989) Acetylcholinesterase prophylaxis against organophosphate poisoning; quantitative correlation between protection and blood-enzyme level in mice. *Biochem. Pharmacol.* 38:529-534.

Raveh, L., Grunwald, J., Marcus, D., Papier, Y., Cohen, E. and Ashani, Y. (1993) Human butyrylcholinesterase as a general prophylactic antidote for nerve agent toxicity; *in vitro* and *in vivo* quantitative characterization. *Biochem. Pharmacol.* 45:2465-2474.

Ripoll, D.R., Faerman, C.H., Axelsen, P.H., Silman, I. and Sussman, J.L. (1993) An electrostatic mechanism for substrate guidance down the aromatic gorge of acetylcholinesterase. *Proc. natl. Acad. Sci. U.S.A.* 90:5128-5132.

Roberts, W.L., Myher, J.J., Kuksis, A., Low, M.G. and Rosenberry, T.L. (1988a). Lipid analysis of the glycoinositol phospholipid membrane anchor of human erythrocyte acetylcholinesterase; palmitoylation of inositol results in resistance to phosphoinositol-specific phospholipase C. *J. biol. Chem.* 263:18766-18775.

Roberts, W.L., Santikarn, S., Reinhold, V.N. and Rosenberry, T.L. (1988b). Structural characterization of the glycoinositol phospholipid membrane anchor of human erythrocyte acetylcholinesterase by fast atom bombardment mass spectronomy. *J. biol. Chem.* 263:18776-18784.

Robertson, R.T. (1993). Acetylcholinesterase of neural development: new tricks for an old dog? *NIPS* 8:266-272.

Razon, N., Soreq, H., Roth, E., Bartal, A. and Silman, I. (1984). Characterization of activities and forms of cholinesterases in human primary brain tumors. *Exp. Neurol.* 84:681-695.

Ruberg, M., Rieger, F., Villageois, A., Bonnet, A.M. and Agid, Y. (1986). Acetylcholinesterase and butyrylcholinesterase in frontal cortex and cerebrospinal fluid of demented and non-demented patients with Parkinson's Disease. *Brain Res.* 362:83-91.

Salte, R., Syvertsen, C., Kjonnoy, M. and Fonnum, F. (1987) Fatal acetylcholinesterase inhibition in salmonids subjected to a routine organophosphate treatment. *Aquaculture* 61:173-179.

Sanyal, R.K. and Khanna, S.K. (1971). Action of cholinergic drugs on motility of spermatozoa. *Fertil. Steril.* 22:356-359.

Saxena, A., Doctor, B.P., Maxwell, D.M., Lenz, D.E., Radic, Z. and Taylor, P. (1993) The role of glutamate-199 in the aging of cholinesterase. *Biochem. biophys. Res. Comm.* 197:343-349.

Schwarz, M., Loewenstein, Y., Glick, D., Liao, J., Norgaard-Pedersen, B. and Soreq, H. (1994) Dissection of successive organophosphorus inhibition and oxime reactivation by human cholinesterase variants. *J. Neurochem.* 63 (Suppl. 1):S80D.

Seidman, S., Ben Aziz-Aloya, R., Timberg, R., Loewenstein, Y., Velan, B., Shafferman, A., Liao, J., Norgaard-Pedersen, B., Brodbeck, U. and Soreq, H. (1994) Overexpressed monomeric human acetylcholinesterase induces subtle ultrastructural modifications in developing neuromuscular junctions of *Xenopus laevis* embryos. *J. Neurochem.* 62:1670-1681.

Shafferman, A., Kronman, C., Flashner, Y., Leitner, M., Grosfeld, H., Ordentlich, A., Gozes, Y., Cohen, S., Ariel, N., Barak, D., Harel, M., Silman, I., Sussman, J.L. and Velan, B. (1992a) Mutagenesis of human acetylcholinesterase: identification of residues involved in catalytic activity and in polypeptide folding. *J. biol. Chem.* 267:17640-17648.

Shafferman, A., Velan, B., Ordentlich, A., Kronman, C., Grosfeld, H., Leitner, M., Flashner, Y., Cohen, S., Barak, D. and Ariel, N. (1992b). Substrate inhibition of acetylcholinesterase: residues affecting signal transduction from the surface to the catalytic center. *EMBO J.* 11:3561-3568.

Shafferman, A., Velan, B., Ordentlich, A., Kronman, C., Grosfeld, H., Leitner, M., Flashner, Y., Cohen, S., Barak, D. and Ariel, N. (1992c) Acetylcholinesterase catalysis - protein engineering studies. In: *Multidisciplinary Approaches to Cholinesterase Functions, Proceedings of the Thirty-Sixth Oholo Conference on Multidisciplinary Approaches to Cholinesterase Functions, Eilat, Israel, 6-10 April 1992*, pp. 165-1754, Shafferman, A,, Velan, B. (eds) Plenum Press, New York.

Shapira, M., Seidman, S., Sternfeld, M., Timberg, R., Kaufer, D., Patrick, J. and Soreq, H. (1994) Transgenic engineering of neuromuscular

junctions in *Xenopus laevis* embryos transiently overexpressing key cholinergic proteins. *Proc. natl. Acad. Sci. U.S.A.* (in press).

Shaw, K.P., Aracava, Y., Akaike, A., Daly, J.W., Rickett, D.L. and Albuqueruqe, E.X. (1985) The reversible cholinesterase inhibitor physostigmine has channel-blocking and agonist effects on the acetylcholine receptor-ion channel complex. *Mol. Pharmacol.* 28:527-538.

Sikorav, J.-L., Duval, N., Anselmet, A., Bon, S., Krejci, E., Legay, C., Osterlund, M., Riemund, B. and Massoulie, J. (1988) Complex alternative splicing of acetylcholinesterase transcripts in *Torpedo* electric organ; primary structure of the precursor of the glycolipid-anchored dimeric form. *EMBO J.* 7:2983-2993.

Silman, I. and Futerman, A.H. (1987). Modes of attachment of acetylcholinesterase to the surface membrane. *Eur. J. Biochem.* 170:11-22.

Silver, A. (1974) *The Biology of Cholinesterases*, North-Holland Publishing Company, Amsterdam.

Soreq, H. and Zakut, H. (1990) *Cholinesterase Genes: Multilevelled Regulation*, Karger, Basel.

Soreq, H. and Zakut, H. (1993) *Human Cholinesterases and Anticholinesterases*, Academic Press, San Diego.

Soreq, H., Malinger, G. and Zakut, H. (1987). Expression of cholinesterase genes in human oocytes revealed by in-situ hybridization. *Human Reproduct.* 2:689-693.

Soreq, H., Seidman, S., Dreyfus, P.A., Zevin-Sonkin, D. and Zakut, H. (1989) Expression and tissue-specific assembly of human butyrylcholinesterase in microinjected *Xenopus* oocytes. *J. biol. Chem.* 264:10608-10613.

Soreq, H., Ben Aziz, R., Prody, C.A., Seidman, S., Gnatt, A., Neville, L., Lieman-Hurwitz, J., Lev-Lehman, E., Ginzberg, D., Lapidot-Lifson, Y. and Zakut, H. (1990). Molecular cloning and construction of the coding region for human acetylcholinesterase reveals a G+C-rich attenuating structure. *Proc. natl. Acad. Sci. U.S.A.* 87:9688-9692.

Soreq, H., Gnatt, A., Loewenstein, Y., Neville, L.F. (1992) Excavations into the active-site gorge of cholinesterases. *Trends biochem. Sci.* 17:353-358.

Soreq, H., Patinkin, D., Lev-Lehman, E., Grifman, M., Ginzberg, D., Eckstein, F., and Zakut, H. (1994) Antisense oligonucleotide inhibition of acetylcholinesterase gene expression induces progenitor cell expansion and suppresses hematopoietic apoptosis *ex vivo*. *Proc. natl. Acad. Sci. U.S.A.* (in press).

Sussman, J.L., Harel, M., Frolow, F., Oefner, C., Goldman, A., Toker, L. and Silman, I. (1991). Atomic structure of acetylcholinesterase from *Torpedo californica*: a prototypic acetylcholine-binding protein. *Science* 253:872-879.

Sussman, J.L., Harel, M. and Silman, I. (1992) Three dimensional structure of acetylcholinesterase. In: *Multidisciplinary Approaches to Cholinesterase Functions, Proceedings of the Thirty-Sixth Oholo Conference on Multidisciplinary Approaches to Cholinesterase Functions, Eilat, Israel, 6-10 April 1992*, pp. 95-108, Shafferman, A,, Velan, B. (eds) Plenum Press, New York.

Taylor, P. (1990) Cholinergic agonists, Anticholinesterase agents. In: *Pharmacological Basis of Therapeutics*, pp. 122-130, 131-149, Gilman, A.G., Rall, T.W., Nies, A.S. and Taylor, P. (eds) Pergamon Press, New York.

Taylor, P. (1991). The cholinesterases. *J. biol. Chem.*266:4025-4028.

Taylor, P. and Lappi, S. (1975) Interaction of fluorescence probes

with acetylcholinesterase: the site and specificity of propidium binding. *Biochemistry* 145:1989-1997.

Taylor, P. and Radic, Z. (1994) The cholinesterases: from genes to proteins. *Annu. Rev. Pharmacol. Toxicol.* 34:281-320.

Toutant, J.P., Massoulie, J. and Bon, S. (1985) Polymorphism of pseudocholinesterase in Torpedo marmorata tissues: comparative study of the catalytic and molecular properties of this enzyme with acetylcholinesterase. *J. Neurochem.* 44:580-592.

Tsim, K.W.K., Randall, W.R. and Barnard, E.A. (1988). An asymmetric form of muscle acetylcholinesterase contains three subunit types and two enzymic activities in one molecule. *Proc. natl. Acad. Sci. U.S.A.* 85:1262-1266.

Turner, A.J. (1994) PIG-tailed membrane proteins. *Essays Biochem.* 28:113-127.

United Nations Security Council (1984) Report of specialist appointed by the Secretary General. Paper S/16433.

Valentino, R.J., Lockridge, O., Eckerson, H.W. and La Du, B.N. (1981) Prediction of drug sensitivity in individuals with atypical serum cholinesterase based on *in vitro* biochemical studies. *Biochem. Pharmacol.* 30:1643-1649.

Velan, B., Grosfeld, H., Kronman, C., Leitner, M., Gozes, Y., Lazar, A., Flashner, Y., Marcus, D., Cohen, S. and Shafferman, A. (1991) The effect of elimination of intersubunit disulfide bonds on the activity, assembly, and secretion of recombinant human acetylcholinesterase: expression of acetylcholinesterase cys-580 → ala mutant. *J. biol. Chem.* 266:23977-23984.

Velan, B., Kronman, C., Ordentlich, A., Flashner, Y., Leitner M., Cohen, S. and Shafferman, A. (1993) N-Glycosylation of human acetylcholinesterase: effects on enzyme activity, stability and biosynthesis. *Biochem. J.* 296:649-656.

Vellom, D.C., Radic, Z., Li, Y., Pickering, N.A., Camp, S. and Taylor, P. (1993). Amino acid residues controlling acetylcholinesterase and butyrylcholinesterase specificity. *Biochemistry* 32:12-17.

Watkins, P.B., Zimmerman, H.J., Knapp, M.J., Gracon, S.I. and Lewis, K.W. (1994) Hepatoxic effects of tacrine administration in patients with Alzheimer's disease. *J. Am. med. Assn.* 271:992-998.

Wecker, L., Kiauta, T. and Dettbarn, W.-D. (1978). Relationship between acetylcholinesterase inhibition and the development of a myopathy. *J. Pharmacol. exp. Ther.* 206:97-104.

Whittaker, M. (1986) *Cholinesterase*, Karger, Basel.

Willems, J.L., DeBisschop, H.C., Verstraete, A.G., Declerck, C. Christiaens, Y. Vanscheeuwyck, P., Buylaert, W.A., Vogelaers, D. and Colardyn, F. (1993) Cholinesterase reactivation in organophosphorus poisoned patients depends on the plasma concentrations of the oxime pralidoxime methylsulphate and of the organophosphate. *Arch. Toxicol.* 67:79-84.

WHO (1986a) Organophosphorus Insecticides: a General Introduction. Environmental Health Criteria 63, World Health Organization, Geneva.

WHO (1986b) Carbamate Pesticides: a General Introduction. Environmental Health Criteria 64, World Health Organization, Geneva.

Wills, J.H. (1970). Toxicity of anticholinesterases and treatment of poisoning. In: *Anticholinesterase Agents, International Encyclopedia of Pharmacology and Therapeutics Section 13*, pp. 357-369, Karczmar, A.G. (ed) Pergamon Press, Oxford.

Winker, M.A. (1994) Tacrine for Alzheimer's disease; which patient, what dose? *J. Am. med. Assn.* 271:1023-1024.

Wolfe, A.D., Blick, D.W., Murphy, M.R., Miller, S.A., Gentry, M.K., Hartgraves, S.L. and Doctor, P.B. Use of cholinesterases as pretreatment drugs for the protection of rhesus monkeys against soman toxicity. *Toxicol. appl. Pharmacol.* (in press)

Wright, C.I., Guela, C. and Mesulam, M.M. (1993) Protease inhibitors and indoleamines selectively inhibit cholinesterases in the histopathologic structures of Alzheimer disease. *Proc. natl. Acad. Sci. U.S.A.* 90:683-686.

Zakut, H., Matzkel, A., Schejter, E., Avni, A. and Soreq, H. (1985) Polymorphism of acetylcholinesterase in discrete regions of the developing human fetal brain. *J. Neurochem.* 45:382-389.

Zakut, H., Ehrlich, G., Ayalon, A., Prody, C.A., Malinger, G., Seidman, S., Ginzberg, D., Kehlenbach, R. and Soreq, H. (1990). Acetylcholinesterase and butyrylcholinesterase genes coamplify in primary ovarian carcinomas. *J. clin. Invest.* 86:900-908.

Zakut, H., Lieman-Hurwitz, J., Zamir, R., Sindell, L., Ginsberg, D. and Soreq, H. (1991) Chorionic villi cDNA library displays expression of butyrylcholinesterase: putative genetic disposition for ecological danger. *Prenatal Diagnosis* 11:597-607.

Legends to Figures

Fig. 1. Cholinesterases and the catalytic process.
A. Catalytic hydrolysis by ChEs the analogous phosphorylation and reactivation processes. Hydrolysis of an acyl-choline substrate (ChO-OCR) by a ChE (EnzOH) proceeds by two steps and involves the production of an acyl-enzyme intermediate (EnzO-OCR). An organophosphorus compound, $XPO(OR')_2$, interacts with these enzymes in a parallel fashion, and may be displaced from the active site serine (S200) by strong nucleophiles (e.g. an oximes, represented as B:), releasing a reactivated enzyme.
B. Protein structure and the main active site domains. A ribbon model of *Torpedo* AChE is presented (after Sussman *et al.*, 1991). Space filling representations of serine 200 (S200) at the active (catalytic) site (AS), aspartate 70 (D70) at the entrance to the active site gorge (ASG), and tryptophan 279 (W279), part of the peripheral anionic site (PAS) are noted.

Fig. 2. Amino acid residues substituted in CHEs from various species.
Schematic view of site-directed mutations introduced into ChEs from different species (human, *Torpedo*, *Drosophila*, and mouse), is shown in a linear scale marked by symbols specific to each enzyme and species. Important residues are noted by numbers and their association with the catalytic triad, hydrophobic subsite, peripheral anionic site (PAS), choline binding site (CBS) and acyl binding site (ABS) is outlined. Note that the *Drosophila* N174S and N331D mutations have no counterpart in the vertebrate enzymes, and therefore do not appear in the figure.

Fig. 3. The human CHE genes and their mRNA transcripts and protein products.
Schematic drawings of the two human genes encoding ChEs is presented. Exons (E) are shaded, introns (I) are white, the ACHE promoter is cross-hatched, and the alternative AChE exon, E5, is black. Open reading frames are marked by dashed underlining, alternative splicing options by triangles over the excised sequences, and constant splicing sites, by solid triangles over the excised sequences.

Fig. 4. Drugs that are substrates or effectors of cholinesterases.

Fig. 5. Organophosphorus cholinesterase inhibitors.
The general formula indicates by X the substituent on the central phosphorus atom that is displaced by the ChE's active site serine hydroxyl group (Fig. 1A). The examples include an insecticide, parathion, a nerve gas, soman, a pharmacological agent, echothiophate, and a cyanobacterial product, anatoxin-A(S).

"	E199D	"	"	Use of carbamoylating substrates demonstrate predominant influence of E199 on acylation rather than deacylation step. k_{cat} decreased 5-fold. K_m for ATCh unchanged. No substrate inhibition	
"	E199H	"	"	Almost complete loss of catalytic activity.	COS1, Gibney *et al.*, 1990
S200	S200V	"	A, B AS.	Triad member. No detectable catalytic activity.	
"	S200C	"	"	0.3% catalytic activity of WT enzyme	
"	S203A	hAChE	"	No detectable catalytic activity. Normally secreted.	Shafferman *et al.*, 1992a, c
"	S203C	"	"		
"	S203T	"	"		
"	S198C	hBuChE	"	1% catalytic activity of WT enzyme	XO, Gnatt *et al.*, 1992, 1994 Soreq *et al.*, 1992
"	S198T	"	"	No detectable catalytic activity.	
"	S198D	"	"		
"	S198H	"	"		
"	S198Q	"	"		
L252	C328V	dChE	a, b	No effect on molecular weight. Not involved in intrachain S-S bonds.	XO, Fournier *et al.*, 1992 Mutero and Fournier, 1992
(-)	N331D	"	(-)	Glycosylation site on 55kd subunit.	XO, Fournier *et al.*, 1992 Mutero and Fournier, 1992
E278	E285A	hAChE	A, B except dChE Rim of ASG.	Only charged residue in PAS. Up to 6-fold increase in K_i for PAS and AS specific ligands.	HEK293 Barak *et al.*, 1994
	Y72A + E285A	"	"	K_i for propidium increased 10-fold.	
W279	W286A	"	A, b Rim of ASG. Core of PAS.	Essential element in intramolecular communication down the gorge; moves upon edrophonium and tacrine binding. K_i for PAS and AS specific ligands increased up to 13-fold.	Shafferman *et al.*, 1992b Barak *et al.*, 1994 Ordentlich *et al.*, 1993a

"	Y72N Y124Q	mAChE	"	Summation of stabilization energies ($\Delta\Delta G$) of single mutations suggests lack of appreciable interaction between the loci. K_i for PAS and AS specific ligands increased up to 100-fold. See also mouse chimera.	HEK293 Radic *et al.*, 1993
D128	D131N	hAChE	Surface.	No effect on activity.	Shafferman *et al.*, 1992c
D172	D175N	"	A, B Part of α-helix C.	Salt bridge with conserved R152. Mutein inactive and not secreted due to misfolding.	
--	N174S	dChE	Glyco-sylation site.	No homologous residue in other ChEs.	XO, Mutero and Fournier, 1992 Fournier *et al.*, 1992
D172	D248N	"	A, B	See mutation in hAChE.	
E199	E202Q	hAChE	A, B AS	Critical residue in AS conformation. Only charged residue at bottom. Stabilizes protonated His447 imidazolium, facilitating proton transfer from S203 and formation of acyl-enzyme intermediate. Hydrogen bond with E450. Mutant is less effective in acylation/deacylation steps. k_{cat} reduced 7-fold. IC_{50} to bisquaternary compounds increased up to 40-fold. Inactivation rate constant reduced 50-fold. Substrate inhibition affected. Confers resistance to aging.	HEK293 Shafferman *et al.*, 1992b, c Ordentlich *et al.*, 1993b
"	E202D	"	"	See 202Q. k_{cat} reduced 25-fold. IC_{50} to edrophonium and bisquaternary compounds increased 25- and up to 80-fold, respectively.	Shafferman *et al.*, 1992b
"	E202A	"	"	see 202Q. k_{cat} reduced 37-fold. IC_{50} to edrophonium and bisquaternary compounds increased 80- and up to 800-fold, respectively.	
"	E199Q	tAChE	"	May interact with E443 and S226 through an entrapped water molecule. Effects interaction with PAS specific inhibitors, suggesting allosteric coupling between the sites. K_m increased 14-fold. k_{cat}/K_m is 2% of that for WT enzyme. Shift in substrate inhibition profile. Mutein largely resistant to aging.	COS1, Gibney *et al.*, 1990 Radic *et al.*, 1992 Saxena *et al.*, 1993

W84	W86A	"	A, B CBS	Element of the hydrophobic subsite and classical anionic site. Methyl group of quaternary ammonium is positioned 4A from Trp indol plane. Involved in stabilization of choline-moiety in the enzyme-substrate or inhibitor-complex. Has a major role in hydrolysis. Motion of residue suggested to be involved in accommodation of ligands. K_m increased 775-fold and k_{cat}/K_m decreased 3890-fold. K_i for propidium and decamethonium increased 621- and 15,000-fold, respectively. Involvement in anionic site identified also in photoaffinity labeling.	HEK293 Barak *et al.*, 1994 Shafferman *et al.*, 1992b, c Ordentlich *et al.*, 1993a
"	W86E	"	"	No detectable catalytic activity. See W86A.	Shafferman *et al.*, 1992b
E89	N126D	dChE	A, B (±)	Glycosylation site on 18kd subunit.	XO, Mutero and Fournier 1992 Fournier *et al.*, 1992
E92	E92Q	tAChE	A, B	Salt bridge between E92 and R44 (both conserved) stabilizing the protein conformation. 1% catalytic activity as compared with WT.	COS1, Bucht *et al.*, 1992
"	E92L	"	"	"	"
D93	D93N	"	A, B Surface.	Hydrogen bond between D93 and Y96. 20% catalytic activity as compared with WT.	COS1, Bucht *et al.*, 1992 HEK293 Shafferman *et al.*, 1992c
"	D95N	hAChE	"	Low production. May be involved in targetting to secretion vesicles. No effect on catalysis.	HEK293 Shafferman *et al.*, 1992a, c
"	D130N	dChE	"	No effect.	XO, Fournier *et al.*, 1992
Y121	Y124A	hAChE	A, B ASG rim.	Part of PAS. Small or no effect on K_m, k_{cat} and propidium binding.	HEK293 Barak *et al.*, 1994
"	Y124Q	mAChE	"	See hAChE Y124A and mAChE Y72N. K_i for PAS and AS specific ligands increased up to 7-fold.	HEK293 Radic *et al.*, 1993
Y70 Y121	Y72A + Y124A	hAChE	"	see single mutations. K_i for propidium and BW284c51 increased 5- and 35-fold, respectively.	HEK293 Barak *et al.*, 1994

"	Y109G	dChE	A, B except in *Drosophila* ASG rim.	Y109 is not directly involved in catalysis but contributes to the AS conformation. Mutations in Y109 produce allosteric effects. Less stable then WT at high temperature. Decreased activity at pH10. K_m for ATCh increased 8-fold.	XO, Mutero *et al.*, 1992 Fournier *et al.*, 1992
"	Y109D	"	"	Km unchanged. Ki for propidium increased 3-fold. Inhibition rate constant for dichlorovos and neostigmine increased 2.5- and decreased 6-fold, respectively.	"
"	Y109K	"	"	Slight changes in inhibition rate constants for OPs and carbamates. More stable than WT at high temperature. K_m for ATCh and BTCh increased 128- and 27-fold, respectively. K_i for propidium increased 205-fold. Inhibition rate constants for paraoxon and dichlorovos decreased 6-fold and increased 4-fold, respectively.	"
"	D74N	mAChE	"	K_m and K_{ss} for ATCh increased 28-and 35-fold, respectively. Dramatic effect on stabilization of BW284C51 and decamethonium complexes, and 680- and 240-fold increases in K_i for these compounds, respectively.	HEK293 Radic *et al.*, 1993
E82	E84Q	hAChE	A, B Surface.	No effect on catalytic properties.	Shafferman *et al.*, 1992a, c

"	W286R	mAChE	:	Contributes to the stabilization energy for PAS and AS specific ligand complexes. K_i for these ligands increased up to 110-fold. See also double, triple and quadruple mutants.	HEK293 Radic *et al.*, 1993
Y70 W279	Y72A + W286A	hAChE	"	Synergistic effect of single mutations. IC_{50} for PAS and AS specific ligands increased up to 130-fold.	all of them???
"	Y72N W286R	mAChE	"	Summation of stabilization energies ($\Delta\Delta G$) of BW284C51 and decamethonium complexes of single mutations, suggests lack of appreciable interaction between the loci. Km (ATCh) and K_i for PAS and AS specific ligands increased 15- and up to 570-fold, respectively.	HEK293 Radic *et al.*, 1993
Y121 W279	Y124Q W286R	"	"	Km (ATCh) and K_i for PAS and AS specific ligands increased 15- and up to 2,500-fold, respectively. See mouse double mutant above.	"
"	E285A + W286A	hAChE	"	Synergistic effect of single mutations. IC_{50} for PAS and AS specific ligands increased up to 222-fold.	all of them???
"	Y72A + E285A + W286A	"	"	Synergistic effect of single mutations. IC_{50} for PAS and AS specific ligands increased up to 387-fold.	all of them???
"	Y124A + E285A + W286A	"	"	Synergistic effect of single mutations. IC_{50} for PAS and AS specific ligands increased up to 350-fold.	all of them???
Y70 Y121 W279	Y72N Y124Q W286R	mAChE	"	Summation of stabilization energies ($\Delta\Delta G$) of BW284C51 and decamethonium complexes of single mutations, suggests lack of appreciable interaction between the loci. K_m (ATCh) and K_i for PAS and AS specific ligands increased 18- and up to 5,700-fold, respectively. K_i of mutein for BW284C51 close to that of BuChE. However, affinity for decamethonium is far lower, indicating that BuChE may use different residues than AChE to stabilize this compound.	HEK293 Radic *et al.*, 1993

"	Y72N Y124Q W286A	"	"	Km (ATCh) and K_i for PAS and AS specific ligands increased 5- and up to 1,500-fold, respectively. See also mouse triple mutant above.	"
Y70 D72 Y121 W279	Y72N D74N Y124Q W286R	"	"	The influence of D74 on the stabilization energies ($\Delta\Delta G$) of BW284C51 and decamethonium complexes is not additive to the triple-mutants. K_m (ATCh) and K_i for PAS and AS specific ligands increased 133- and up to 280,000-fold, respectively.	"
Y70 D72 Y121 W279	Y72N D74N Y124Q W286A	"	"	K_m (ATCh) and K_i for PAS and AS specific ligands increased 46- and up to 60,000-fold, respectively. See quadruple mutant above.	"
"	W279A	tAChE	"	See human AChE. Conformational changes upon binding of tacrine, edrophonium. 10-fold decrease in sensitivity to propidium. No change in binding of edrophonium. Slightly less inhibited by high substrate concentrations.	Harel *et al.*, 1992
F288	F295L	hAChE	A, B ASG bottom; part of the ABS	Important in determining specificity to acyl moiety of substrate. Improved interaction with BTCh in the mutants. Increased affinity to iso-OMPA due to substitution with smaller residue. K_m for BTCh 10-fold reduced. K_m for ATCh unchanged.	HEK293, Ordentlich *et al.*, 1993a
"	F295A	"	"	K_m for BTCh 30-fold reduced. K_i for BW284C51 3-fold reduced.	Barak *et al.*, 1994 Ordentlich *et al.*, 1993a
"	F295L	mAChE	"	Reduced k_{cat} for ATCh. Enhanced k_{cat} and reduced K_m for BTCh.	HEK293, Vellom *et al.*, 1993 293 Radic *et al.*, 1993
"	F295Y	"	"	K_m (ATCh) and k_{cat} decreased 2.5- and 25-fold respectively.	HEK293 Radic *et al.*, 1993
"	L286D	hBuChE	"	Catalytic activity significantly affected. K_m increased 4-fold. Reduced sensitivity to a variety of OPs. IC_{50} for echothiophate increased 9-fold.	XO, Gnatt *et al.*, 1994 Schwarz *et al* , 1994
"	L286Q	"	"	K_m increased 10-fold.	"

"	L286R	"	"	K_m increased 5-fold.	"
"	L286K	"	"	K_m increased 10-fold. IC_{50} for echothiophate increase more than 1,000-fold. Reactivation rate of DIP-BuChE by 2-PAM decreased 40-fold.	"
"	F288L + F290V	tAChE	ABS	Removal of bulky phenyl rings appears to permit BCh to enter the active site, and allows space for the entrance of bulky OPs such as iso-OMPA. Specific activity with ATCh as substrate is 10% of WT enzyme. BTCh hydrolyzed at same rate as ATCh.	COS 7, Harel *et al.*, 1992
R289	R296S	mAChE	A, B	No major change in catalytic parameters. Slight decrease in K_m for BTCh due to reduced steric hindrance.	HEK293, Vellom *et al.*, 1993 HEK293 Radic *et al.*, 1993
F290	F297V	hAChE	A, B (±) ASG bottom; part of ABS	Secondary to F295 in determining specificity to acyl-moeity of substrate. Contributes to AS plasticity. K_m for ATCh increased 9-fold.	HEK293, Ordentlich *et al.*, 1993a
"	F297A	"	"	See F297V. Affinity to BW284C51 increased 2-fold. K_i for decamethonium increased 10-fold. K_m for ATCh increased 4-fold.	Barak *et al.*, 1994 Ordentlich *et al.*, 1993a
"	F297I	mAChE	"	Eliminates substrate inhibition and confers substrate activation. Role in orienting substrate to maximize catalysis. Reduced k_{cat} for ATCh but increased k_{cat} for BTCh. K_m for ATCh increased 9-fold, but K_m for BTCh unchanged.	HEK293, Vellom *et al.*, 1993 HEK293 Radic *et al.*, 1993
"	F297Y	"	"	K_m for ATCh unchanged.	HEK293 Radic *et al.*, 1993
"	F295L + F297V	hAChE	"	See single mutations.	Ordentlich *et al.*, 1993a
"	F295L + F297I	"	"	See F297.	HEK293, Vellom *et al.*, 1993 HEK293 Radic *et al.*, 1993
"	F295L + R296S + F297I	"	"	Lower k_{cat} for ATCh as compared with single mutations. 5% activity of WT enzyme.	HEK293, Vellom *et al.*, 1993
V293	V300G	mAChE	A, B Near ABS.	See R296S.	HEK293, Vellom *et al.*, 1993 Radic *et al.*, 1993

D326	D333N	hAChE	A, B ASG bottom	Charged moiety pointing away from triad. No effect on catalysis, but secretion reduced.	Shafferman *et al.*, 1992a, c
E327	E334Q		A, B AS	Triad member. No detectable activity.	
"	E334A	"	"	"	
"	E334D	"	"	"	
F330	Y337F	"	A (±), B AS.	Element of hydrophobic subsite. Minimal contribution to phosphorylation and dealkylation of OP-enzyme conjugate. Signal received from Y341 induces conformational changes (observed rotation of aryl moiety into gorge cavity upon edrophonium/tacrine binding), interfering/preventing formation of ACh-enzyme complex causes substrate inhibition. Substrate inhibition not affected by mutation. IC_{50} for PAS and AS specific ligands increased up to 15-fold.	Shafferman *et al.*, 1992b Barak *et al.*, 1994 Ordentlich *et al.*, 1993a
"	Y337A	"	"	No substrate inhibition due to substitution with small residue. K_i to edrophonium increased 8 to 30-fold. K_i to BW284C51 increased 50-fold. K_i to decamethonium decreased 7-fold.	Ordentlich *et al.*, 1993a, b Shafferman *et al.*, 1992b, c Barak *et al.*, 1994
"	Y337A	mAChE	"	K_i for substituted phenothiazines is up to 1,800-fold higher in AChE than BuChE. This difference is due primarily to Y337A substitution. Affinity for tacrine increased 7-fold, but decreased 19-fold for edrophonium, suggesting a role for this residue in stabilizing the latter but not the former ligand.	HEK293 Radic *et al.*, 1993
"	Y337F	"	"	Little influence on K_i for substituted phenothiazines. Affinity for tacrine decreased 3-fold.	"
F331	F338A	hAChE	A, B Near AS.	Element of the hydrophobic subsite. Contributes to AS plasticity. K_i to decamethonium increased 3-fold.	Ordentlich *et al.*, 1993a Shafferman *et al.*, 1992b, c Barak *et al.*, 1994
"	F338G	mAChE	"	Little influence on K_i for substituted phenothiazines.	HEK293 Radic *et al.*, 1993

"	F329Q	hBuChE	"	See above. K_m unchanged. Changes in interaction with OPs depending on nature of both OP and residue substituted. IC_{50} to iso-OMPA decreased 10-fold.	XO, Gnatt *et al.*, 1994 Schwarz *et al.*, 1994 Soreq *et al.*, 1992
"	F329L	"	"	IC_{50} to iso-OMPA and echothiophate increased 10 and 14-fold, respectively.	
"	F329C	"	"	See F329L. Reactivation rate of DIP-BuChE by 2-PAM decreased 10-fold.	
"	F329D	"	"	See F329L. Reactivation rate of DIP-BuChE by 2-PAM decreased 19-fold.	
Y334	Y341A	hAChE	A, B ASG rim (close to D74).	Relays signal from D74 to Y337. Can form hydrogen bond with D74. Effect on the stability of the enzyme complex with BW284C51 and decamethonium. K_i for PAS and AS specific inhibitors increased up to 34-fold. Substrate inhibition affected.	HEK293, Shafferman *et al.*, 1992b Barak *et al.*, 1994
D342	D349N	"	A, B Surface.	No effect on catalysis, but secretion reduced.	Shafferman *et al.*, 1992a, c
D397	D404N	"	A, B Salt bridge.	Forms hydrogen bond with Y382 and salt bridge with R525. Crucial stabilizing role bringing together C409 and C382 (S-S loop). No detectable activity and no secretion.	
H425	H425Q	tAChE	A, B Near AS.	50% activity of WT enzyme. Mutation proved this residue is not a triad member.	COS 1, Gibney *et al.*, 1990
"	H432A	hAChE		Secretion reduced.	HEK293, Shafferman *et al.*, 1992a, c
I439	M437D	hBuChE	A (±), B Near AS.	No detectable activity suggests structural role.	XO, Gnatt *et al.*, 1992 Gnatt *et al.*, 1994 Soreq *et al* ., 1992
H440	H440Q	tAChE	A, B AS.	Triad member. No detectable activity.	COS 1, Gibney *et al.*, 1990
"	H425Q H440Q	"	"	No detectable activity.	

"	H447A	hAChE	"	"	HEK293, Shafferman *et al.*, 1992a, c
Y442	Y440D	hBuChE	A, B except dChE	Normal activity. Moderately decreased OP-binding.	XO, Neville *et al.*, 1992 Gnatt *et al.*, 1992 Gnatt *et al.*, 1994 Soreq *et al.*, 1992
E443	E450A	hAChE	A, B Near AS.	Hydrogen bonded with E202. Effect of mutation interactions with DFP, soman and substrates are similar to those of E202Q, due to hydrogen bond between these residues. Inactivation rate constant for DFP decreased 100-fold. Mutein shows marked resistance to aging.	HEK293, Ordentlich *et al.*, 1993b
E443 E445	E441G E443Q	hBuChE	A, B Near AS.	3% catalytic activity of WT enzyme. Defective substrate H_2O-binding.	XO, Neville *et al.*, 1992 Soreq *et al.*, 1992
V453	N531D	dChE	A (±), B(±)	Glycosylation site on 55kd subunit.	Fournier *et al.*, 1992 Mutero and Fournier, 1992
L495	N569S	"	A, B	"	Mutero and Fournier, 1992
I537	C615R	"	a, b	Not involved in intrachain S-S bonds. Involved in intersubunit S-S bond. Smaller protein migrating as a monomer (75kd).	Fournier *et al.*, 1992 Mutero and Fournier, 1992
C572	C580A	hAChE	A, B	Causes secretion as monomer, suggesting that subunit assembly is not coupled to transport. No change in catalytic properties.	HEK293, Velan *et al.*, 1991
"	C580S	"	"	20-40 fold increase in specific activity, but no change in catalytic properties. Mutation facilitate correct folding.	*E. coli*., Fischer *et al.*, 1993

Chimeras and deletions.		
mBuCHE 5-174 in mAChE.	Selectivity to ethropropazine and iso-OMPA dictated by domains 175-487 (increased binding to former and AChE-like binding to latter). Selectivity to BW284C51 is accounted for within 5-174 as seem from major increase in K_i, or more specifically by Y72 and Y124 as evident from the similar value of stabilization energy of the BW284C51 complex of the chimera and the double mutant Y72N, Y124Q.	HEK293, Vellom *et al.*, 1993
mBuCHE 5-174 and 488-575 in mAChE.	See mouse-chimera above.	
hAChE 62-138 in hBuChE. Rim of ASG, conserved CBS and oxyanion hole.	K_m (BTCh) and inhibition by SucCh and physotigmine similar to WT BuChE. Sensitivity to iso-OMPA and echothiophate is AChE-like. Pattern of interaction with bambuterol, dibucaine and BW284C51 is intermediate of AChE and BuChE. Loss of substrate activation. No substrate inhibition.	XO, Loewenstein *et al.*, 1993
Deletion of residues 148-166. in dChE. Cleavage site.	Partial effect on cleavage of protein into two subunits.	Fournier *et al.*, 1992
Deletion of residues 167-180 "	"	"
Deletion of residues 148-180 "	Prevents cleavage.	"

[a]As is conventional (Massoulie *et al.*, 1992), the sequence number of each mutated residue is referred to the homologous residue in *Torpedo* AChE. [b]The prefixes d, h, m and t refer to *Drosophila*, human, mouse and *Torpedo*, respectively. [c]A, B, conserved residue in AChE and BuChE, respectively; a, b, non-conserved residue. A (±), B (±), conserved residue in ChEs of some, but not all, species reported. [d]HEK293, human embryonic kidney cell line 293; XO, *Xenopus* oocytes.

Fig. 1B.

Catalysis

$$ChO\text{-}OCR + EnzOH \longrightarrow EnzO\text{-}OCR + ChOH$$

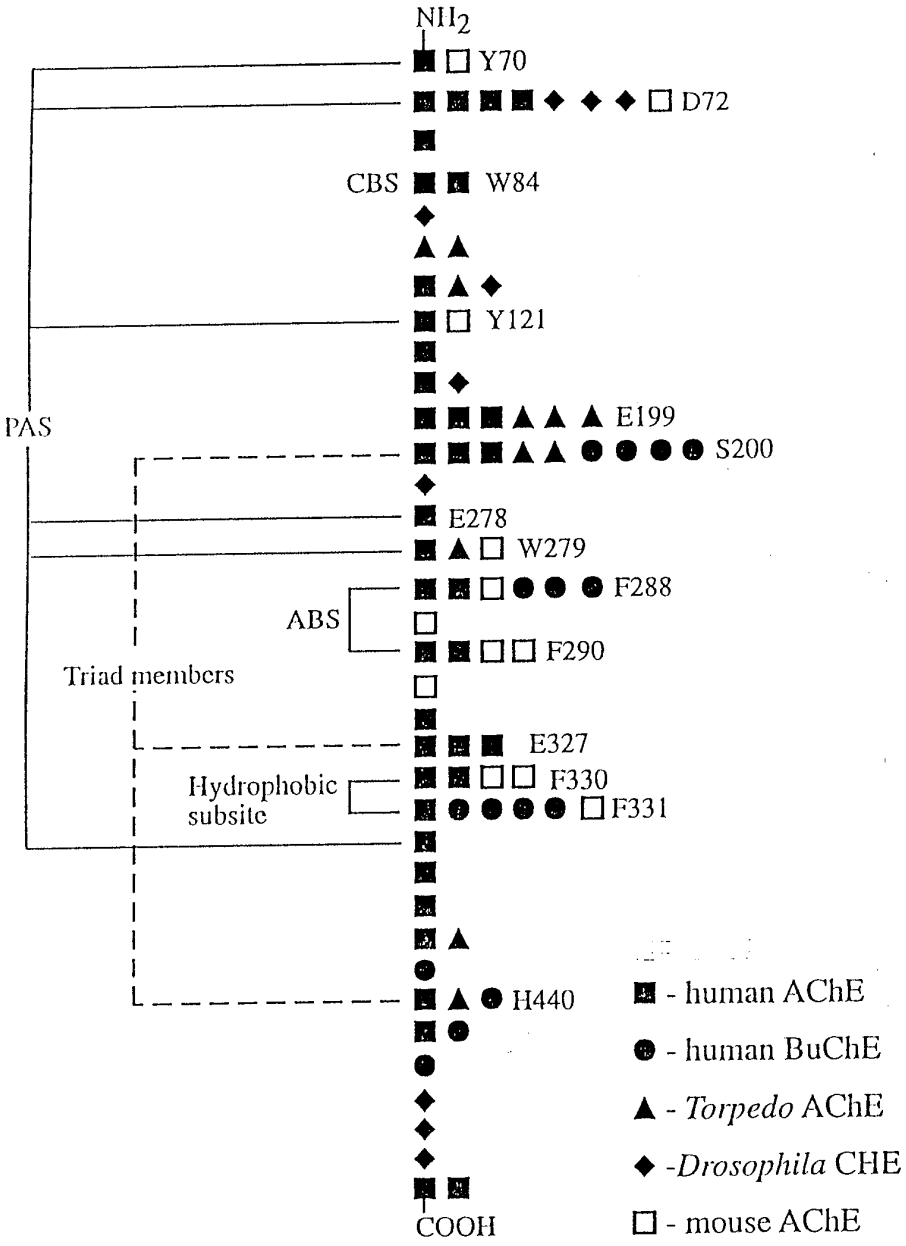
$$EnzO\text{-}OCR + OH^- \longrightarrow {}^-OOCR + EnzOH$$

Phosphorylation and reactivation

$$XPO(OR')_2 + EnzOH \longrightarrow EnzOPO(OR')_2 + HX$$

$$EnzOPO(OR')_2 + B: \longrightarrow B\text{-}PO(OR')_2 + EnzOH$$

Fig. 2. Site-directed amino-acid residue substitutions in ChEs from various species.



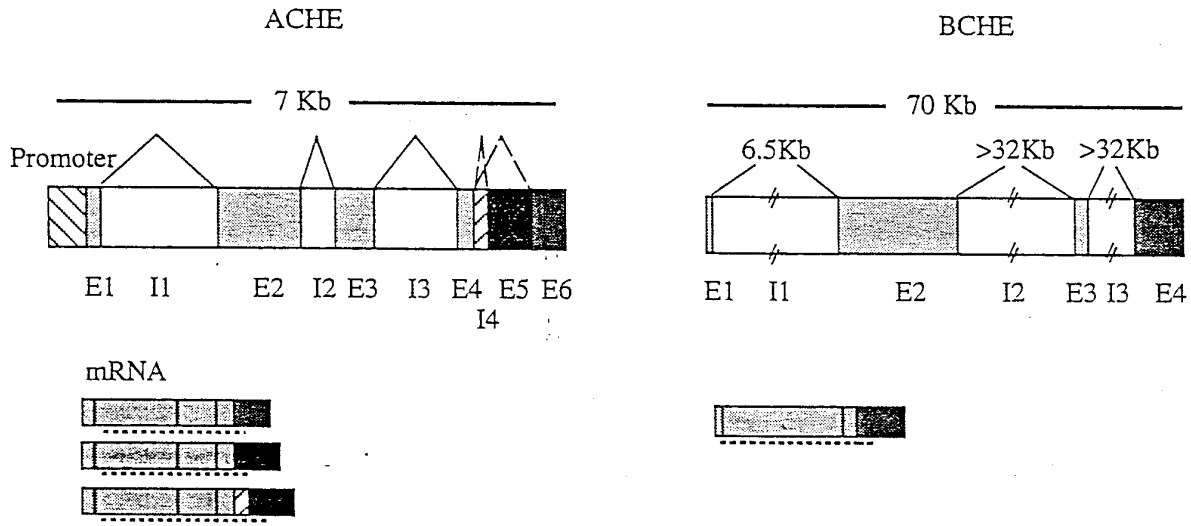
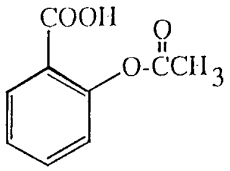
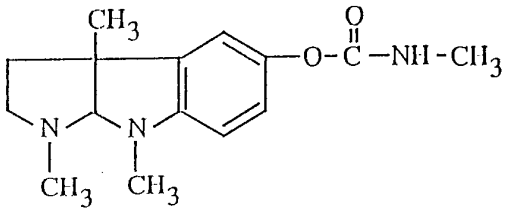

ACHE
7 Kb
Promoter
E1 I1 E2 I2 E3 I3 E4 E5 E6
I4
mRNA
BCHE
70 Kb
6.5Kb
>32Kb
>32Kb
E1 I1 E2 I2 E3 I3 E4

Fig. 3.

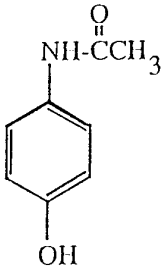
Fig. 4. Drugs that are substrates or effectors of cholinesterases.



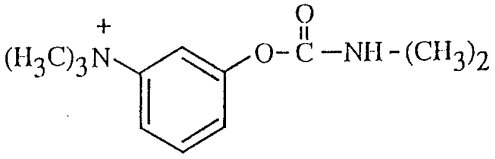
Aspirin



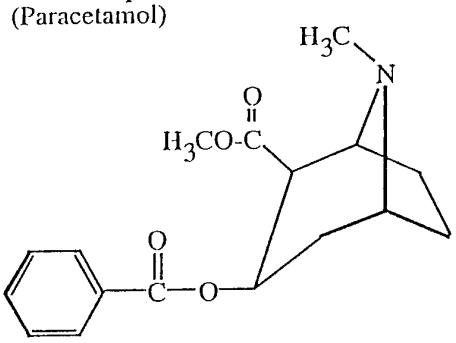
Physostigmine



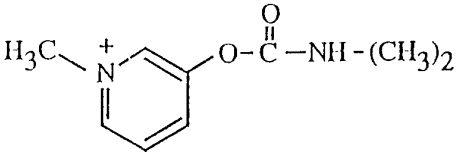
Acetaminophen
(Paracetamol)



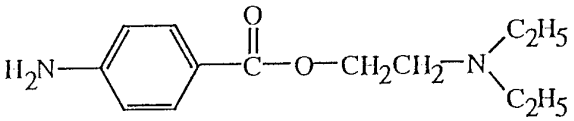
Neostigmine



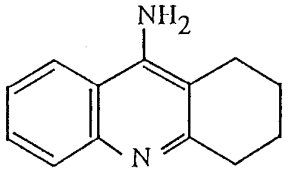
Cocaine



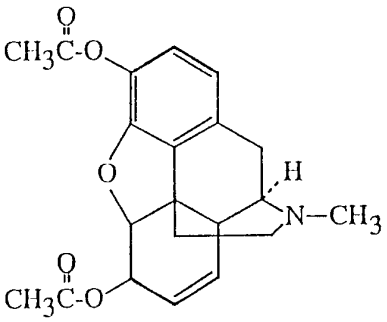
Pyridostigmine



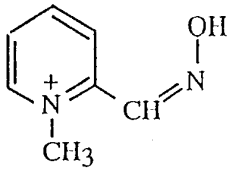
Procaine



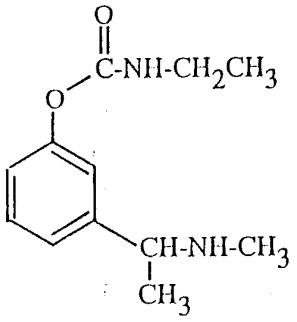
Tacrine



Heroin

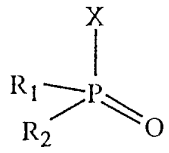


2-PAM

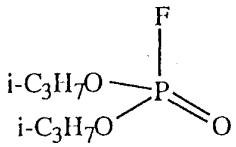


SDZ-ENA713

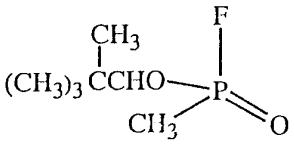
Fig. 5. Organophosphorus ChE inhibitors.



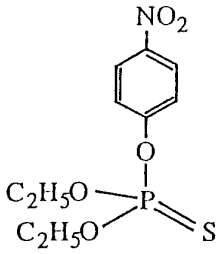
General formula



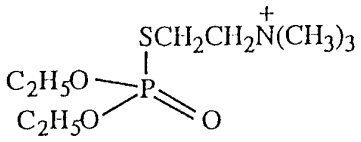
DFP, Diisopropylfluorophosphonate



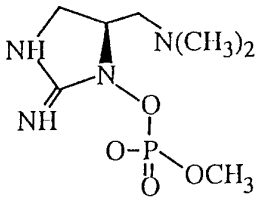
Soman



Parathion



Echothiophate



Anatoxin-A(S)

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Raven Press, Ltd., New York

Overexpressed Monomeric Human Acetylcholinesterase Induces Subtle Ultrastructural Modifications in Developing Neuromuscular Junctions of *Xenopus laevis* Embryos

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Abstract: Formation of a functional neuromuscular junction (NMJ) involves the biosynthesis and transport of numerous muscle-specific proteins, among them the acetylcholine-hydrolyzing enzyme acetylcholinesterase (AChE). To study the mechanisms underlying this process, we have expressed DNA encoding human AChE downstream of the cytomegalovirus promoter in oocytes and developing embryos of *Xenopus laevis*. Recombinant human AChE (rHAChE) produced in *Xenopus* was biochemically and immunochemically indistinguishable from native human AChE but clearly distinguished from the endogenous frog enzyme. In microinjected embryos, high levels of catalytically active rHAChE induced a transient state of overexpression that persisted for at least 4 days postfertilization. rHAChE appeared exclusively as nonassembled monomers in embryos at times when endogenous *Xenopus* AChE displayed complex oligomeric assembly. Nonetheless, cell-associated rHAChE accumulated in myotomes of 2- and 3-day-old embryos within the same subcellular compartments as native *Xenopus* AChE. NMJs from 3-day-old DNA-injected embryos displayed fourfold or greater overexpression of AChE, a 30% increase in postsynaptic membrane length, and increased folding of the postsynaptic membrane. These findings indicate that an evolutionarily conserved property directs the intracellular trafficking and synaptic targeting of AChE in muscle and support a role for AChE in vertebrate synaptogenesis.
Key Words: Neuromuscular junction—*Xenopus laevis* embryos—Human acetylcholinesterase—Muscle.
J. Neurochem. **62,** 1670–1681 (1994).

Formation of a functional neuromuscular junction (NMJ) requires the targeted deposition of synaptic proteins at the nerve–muscle interface (Flucher and Daniels, 1989; Froehner, 1991; Ohlendieck et al., 1991). Among these proteins is the acetylcholine-hydrolyzing enzyme acetylcholinesterase (AChE), responsible for terminating cholinergic neurotransmission across the NMJ (Hall, 1973). It is not yet clear how the selective accumulation of synapse-specific proteins at the NMJ is accomplished. One mechanism for achieving synaptic localization of AChE is probably the compartmentalized transcription and translation of AChE mRNA (Rotundo, 1990; Rossi and Rotundo, 1992). Aggregation and anchoring of AChE at the postsynaptic cell surface are mediated by evolutionarily conserved components of the synaptic basal lamina, such as heparan sulfate proteoglycans (Brandan et al., 1985) and agrin (McMahan, 1990). Similar mechanisms appear to be involved in the biosynthesis of the nicotinic acetylcholine receptor (AChR) (Changeux, 1991; Phillips et al., 1991; Wallace, 1991) and may represent a general solution to the problem of localizing junctional proteins in muscle cells (Pavlath et al., 1989; Ralston and Hall, 1989). However, the molecular determinants controlling the intracellular transport of AChE and the elements specifying its synaptic localization in muscle remain to be elucidated.

In the developing *Xenopus laevis* embryo, muscle differentiation, primitive neuromuscular contacts, and spontaneous synaptic activity are observed within 1 day postfertilization (PF) (Kullberg et al., 1977). During the ensuing 24 h, ultrastructural specializations characterizing synaptic differentiation are observed, followed by the acquisition of spontaneous

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Abbreviations used: AChE, acetylcholinesterase; ACHE, acetylcholinesterase gene; AChR, acetylcholine receptor; CMV, cytomegalovirus; CMVACHE, acetylcholinesterase cDNA downstream of the cytomegalovirus promoter–enhancer element; mAb, monoclonal antibody; NMJ, neuromuscular junction; PF, postfertilization; rHAChE, recombinant human acetylcholinesterase.

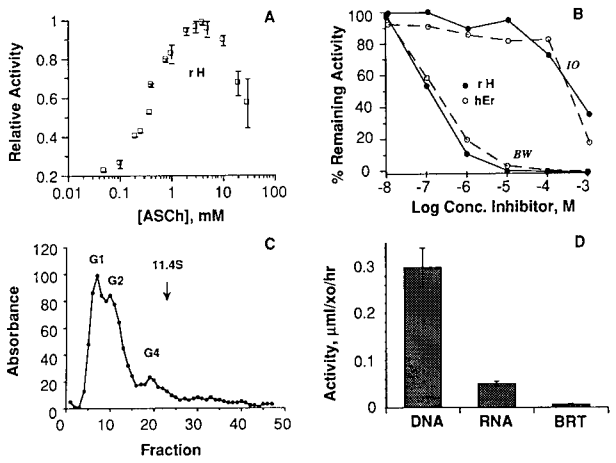

FIG. 1. *Xenopus* oocytes express catalytically active rHAChE. **A:** Inhibition by excess substrate. Mature *Xenopus* oocytes were injected with 5 ng of in vitro-transcribed AChE mRNA (Soreq et al., 1990) and incubated overnight at 17°C. Homogenates corresponding to one-third oocyte were assayed for AChE activity in the presence of various concentrations of acetylthiocholine (ASCh) substrate [average of three experiments ± SEM (bars)]. **B:** Sensitivity to selective inhibitors. Oocyte homogenates were preincubated for 30 min in assay buffer containing the AChE-specific, reversible inhibitor 1,5-bis(4-allyldimethylammoniumphenyl)-pentan-3-one dibromide [BW284C51 (BW)] or the butyrylcholinesterase-specific inhibitor tetraisopropylpyrophosphoramide (IO) at the indicated concentrations and assayed for remaining activity following addition of 2 m*M* ASCh. Data are averages of duplicate assays from two independent microinjection experiments. AChE extracted from human erythrocytes (hEr) served as control. rH, rHAChE. **C:** Oligomeric assembly. Homogenates from ACHE mRNA-injected oocytes were subjected to sucrose density centrifugation as described in Materials and Methods. Data are averages of three experiments. Note that in addition to the free monomer (3.2S; G1), the oocyte appears to generate dimers (5.6S; G2) and to a lesser extent tetramers (10.2S; G4) of human AChE. Endogenous oocyte AChE activity is undetectable under these conditions. The arrow marks the position of bovine liver catalase (11.4S). **D:** Expression of ACHE DNA in *Xenopus*. Oocytes were injected with 5 ng of synthetic ACHE mRNA or CMVACHE (Velan et al., 1991*a*) and incubated for 1 (RNA) to 3 (DNA) days. Oocytes injected with incubation medium (BRT) or uninjected oocytes served as control. Activity is expressed as micromoles of substrate hydrolyzed per hour per oocyte, in mean ± SEM (bars) values for three independent microinjection experiments.

motor activity and hatching (Cohen, 1980). Fervent embryonic development and ultrastructural maturation of the neuromuscular system continue, giving rise to a free swimming tadpole within 4–5 days. From day 2 PF, a steady increase in AChE activity is observed (Gindi and Knowland, 1979), concomitant with a developmentally regulated decrease in the time course of the synaptic potential in *Xenopus* myotomes (Kullberg et al., 1980). The rapid development of the neuromuscular system in *Xenopus* thus makes it an excellent in vivo model for the study of vertebrate myogenesis and synaptogenesis.

We have cloned a DNA sequence encoding human AChE and used it to express catalytically active AChE in microinjected *Xenopus* oocytes (Soreq et al., 1990) and cultured human cells (Velan et al., 1991*a*). Placed downstream of either the native human AChE gene (ACHE) promoter or the cytomegalovirus (CMV) enhancer–promoter and introduced into fertilized *Xenopus* eggs, this DNA led to overexpression of AChE in NMJs of 2-day-old embryos (Ben Aziz-Aloya et al., 1993). Here, we present a biochemical and histochemical characterization of this recombinant human AChE (rHAChE) as expressed in *Xenopus*. Moreover, we demonstrate the persistence of overexpressed enzyme in NMJs to at least day 3 of embryonic development and offer evidence indicating subtle alterations in the ultrastructure of NMJs from embryos overexpressing rHAChE. Our findings indicate the assignment of catalytically active monomeric rHAChE to subcellular compartments common to those occupied by native, multimeric *Xenopus* AChE in embryonic myotomes and suggest a morphogenetic role for AChE in vertebrate synaptogenesis.

MATERIALS AND METHODS

In vitro fertilization and microinjections

DNA microinjections into *X. laevis* oocytes and fertilized eggs were essentially as previously described (Ben Aziz-

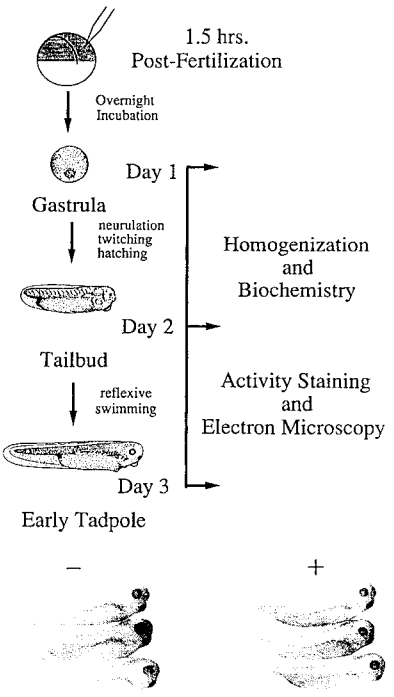

FIG. 2. Normal development of CMVACHE-injected embryos. A schematic representation of a microinjection experiment depicting the principal developmental stages and analytical approaches used in this work is shown together with photographs displaying the normal gross development of unstained microinjected embryos (+) compared with control uninjected embryos (–) 3 days PF. In vitro fertilized eggs of *Xenopus laevis* were injected with 1 ng of CMVACHE and cultured for 1–4 days as described in Materials and Methods. Sketches are modeled after those of Deuchar (1966).

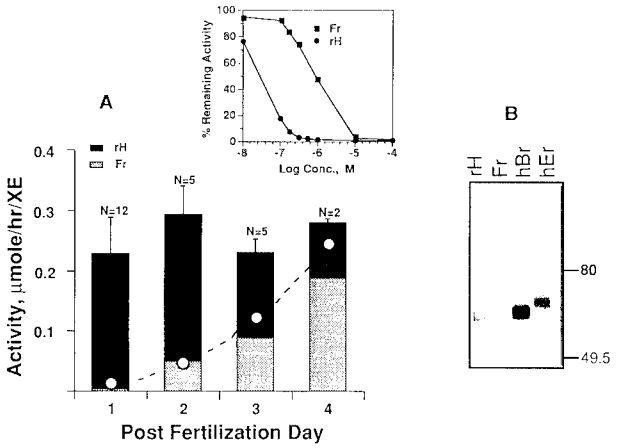

FIG. 3. CMVACHE-injected *Xenopus* embryos express and maintain biochemically distinct heterologous human AChE for at least 4 days. **A:** Overexpression of rHAChE in developing embryos. High-salt/detergent extracts of CMVACHE-injected and uninjected embryos were prepared and assayed for AChE activity in the presence and absence of the selective inhibitor echothiophate (3.3×10^{-7} *M*; **inset**). Endogenous AChE activity was calculated according to an algorithm assuming 90% inhibition of rHAChE and 20% inhibition of frog AChE at this concentration of inhibitor. The bar graph represents the total AChE activity measured per microinjected embryo at various time points following microinjection and the calculated activities attributable to rHAChE (dark shading) and endogenous frog AChE (light shading). The total AChE activity measured in uninjected control embryos at the same time points is indicated by open circles. Data represent average ± SEM (bars) values from four to six embryos from the indicated number (N) of independent microinjection experiments. Inset: Selective inhibition of rHAChE by echothiophate. Homogenates representing endogenous frog (Fr) or recombinant human (rH) AChE were assayed for activity following a 40-min preincubation with the indicated concentrations of echothiophate. Data are averages of three experiments. **B:** Immunochemical discrimination between rHAChE and embryonic *Xenopus* AChE. Affinity-purified AChE from CMVACHE-injected *Xenopus* embryos (rH), control uninjected embryos (Fr), human brain (hBr), and erythrocytes (hEr) was subjected to denaturing gel electrophoresis and protein blot analysis as described in Materials and Methods. Each lane represents ~20 ng of protein, except rH, which contained only 6 ng. Note the complete absence of immunoreactivity with embryonic *Xenopus* AChE, although silver staining of a parallel gel demonstrated detectable protein at the corresponding position (data not shown). The faint upper bands (140–160 kDa) in the lanes displaying native human AChEs represent dimeric forms resulting from incomplete reduction of the intersubunit disulfide bonds (see Liao et al., 1992). Prestained molecular weight markers indicated on the right were from Bio-Rad, U.S.A..

Aloya et al., 1993). Fertilized eggs were dejellied with 2% cysteine and injected within the first two cleavage cycles in medium containing 5% Ficoll in 0.3× Mark's modified Ringer (MMR). Several hours after microinjection, embryos were transferred into 0.3× MMR and cultured overnight at 17–19°C. One-day-old embryos were transferred to either 0.1× MMR or aged tap water and cultured for an additional 1–3 days.

Activity assays

Embryos were harvested in groups of three to five apparently normal individuals and stored frozen until used. Homogenates were prepared in a high-salt/detergent buffer [0.01 *M* Tris, 1.0 *M* NaCl, 1% Triton X-100, and 1 m*M* EGTA (pH 7.4); 150 μl per embryo] and assayed for enzymatic activity as detailed elsewhere (Neville et al., 1992). For subcellular fractionations, groups of three embryos were homogenized in low-salt buffer [0.02 *M* Tris-HCl (pH 7.5), 0.01 *M* $MgCl_2$, and 0.05 *M* NaCl; 100 μl per embryo] and centrifuged at 100,000 rpm for 10 min in a Beckman model TL100 tabletop ultracentrifuge. The supernatant was collected and considered the low-salt-soluble fraction. The pellet was resuspended in low-salt/detergent buffer [0.01 *M* phosphate buffer (pH 7.4) and 1% Triton X-100], incubated on ice for 1 h, and centrifuged as above for 5 min to generate the detergent-soluble fraction. The remaining pellet was resuspended in high-salt buffer [0.01 *M* phosphate buffer (pH 7.4), 1.0 *M* NaCl, and 1 m*M* EGTA] to release the high-salt-soluble AChE fraction.

Protein blot analyses

rHAChE was purified by affinity chromatography from ~180 "day 1" embryos injected with plasmid DNA carrying AChE cDNA downstream of the CMV promoter–enhancer element (CMVACHE) using a modified procedure for the purification of native human AChE (Gennari and Brodbeck, 1985). In brief, AChE from embryos homogenized in low-salt/detergent buffer was bound to Sepharose beads carrying *N*-(1-amino-hexyl)-3-dimethylethylaminobenzoic amide by shaking overnight at room temperature. Elution was with 0.02 *M* edrophonium chloride (Tensilon; Hoffmann-La Roche, Basel, Switzerland). Embryonic *Xenopus* AChE was similarly purified from 1-week-old tadpoles but had to be eluted by boiling in 0.1% sodium dodecyl sulfate. Denaturing sodium dodecyl sulfate–polyacrylamide gel electrophoresis and blotting were essentially as described elsewhere (Liao et al., 1992) using a pool of monoclonal antibodies (mAbs; 132-1,2,3; 6 μg/ml each) raised against denatured human brain AChE (Brodbeck and Liao, 1992).

Sucrose gradient analysis of AChE subunit assembly

Freshly prepared, high-salt/detergent extracts from one or two embryos or five to 10 oocytes were applied to 12-ml 5–20% linear sucrose density gradients and centrifuged overnight at 4°C. Fractions were collected into 96-well mi-

TABLE 1. *Subcellular fractionation of rHAChE in CMVACHE-injected* Xenopus *embryos*

	rH			
Fraction	Day 1	Day 2	Day 3	Fr (day 3)
LSS	57 ± 2	60 ± 4	53 ± 3	36 ± 5
DS	37 ± 2	34 ± 4	36 ± 3	31 ± 4
HSS	6 ± 2	5 ± 1	10 ± 1	33 ± 7

Fertilized *Xenopus* eggs were microinjected with 1 ng of CMVACHE DNA, cultured for 1–3 days, and subjected to homogenization and subcellular fractionation as described in Materials and Methods. rHAChE in each fraction (rH) was detected by enzyme-antigen immunoassay (Liao et al., 1992) using a specific mAb (101-1) raised against bovine brain AChE. Endogenous AChE activity in uninjected tadpoles (Fr) was determined by the standard colorimetric assay described in Materials and Methods. Percent enzyme activity in each fraction (average ± SEM) is shown for three to five groups of three embryos from a single microinjection experiment. LS, low-salt soluble; DS, detergent soluble; HSS, high-salt soluble.

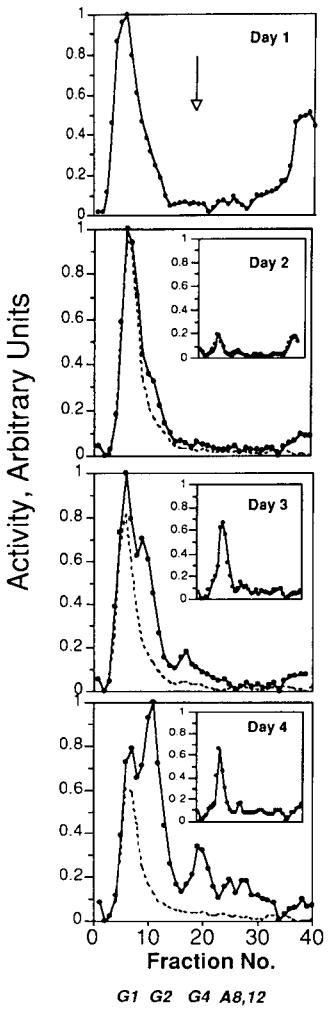

FIG. 4. rHAChE in microinjected *Xenopus* embryos remains monomeric. High-salt/detergent extracts representing two embryos were subjected to sucrose density centrifugation as described in Materials and Methods. Shown are total AChE (solid line) and immunoreactive rHAChE (dotted line) from CMVACHE-injected embryos 1–4 days PF. rHAChE appeared exclusively as a peak representing monomeric AChE (~3.2S) at all time points. The arrow marks the position of bovine liver catalase (11.4S). **Insets:** AChE molecular forms in control uninjected embryos scaled to the total activity levels observed in DNA-injected embryos (see Fig. 2). Peak analysis demonstrated that the distribution of oligomeric forms was identical to that observed in CMVACHE-injected embryos. Note that monomeric AChE is essentially undetectable in control embryos. G1, G2, and G4 indicate the expected positions of the globular monomer, dimer, and tetramer in the gradient; A8 and 12 indicate the positions of "tailed" asymmetric forms. Fraction 0 represents the top of the gradient.

crotiter plates and assayed for total AChE activity as previously described (Soreq et al., 1989). To distinguish between rHAChE and endogenous *Xenopus* AChE in gradient fractions, 100-μl aliquots were transferred to a Maxisorp immunoplate (Nunc, Copenhagen, Denmark) coated with an mAb (mAb 101-1) recognizing human but not frog AChE and diluted 1:1 with double distilled water. Following overnight incubation, the plates were washed three times with phosphate-buffered saline containing 0.05% Tween 20, and each well was assayed for catalytically active AChE.

Cytochemical AChE staining and electron microscopy

Embryos were fixed, cytochemically stained for AChE, and prepared for electron microscopy as previously described (Ben Aziz-Aloya et al., 1993). Cytochemical staining (Karnovsky, 1964) was carried out in acetate buffer (pH 6.1) for 15–20 min at 4°C within 3 days of fixation.

RESULTS

Xenopus-expressed AChE is biochemically indistinguishable from native human AChE

Microinjected into mature *X. laevis* oocytes, 5 ng of in vitro-transcribed AChE mRNA directed the production of catalytically active AChE displaying substrate and inhibitor interactions characteristic of the native human enzyme (Fig. 1A and B). The apparent K_m calculated for rHAChE toward acetylthiocholine was 0.3 m*M*, essentially identical to that displayed by rHAChE expressed in cell lines (Velan et al., 1991*a*) and native human erythrocyte AChE (data not shown). In sucrose density centrifugation rHAChE sedimented primarily as monomers and dimers, although a discernible peak apparently representing globular tetrameric AChE was also observed (Fig. 1C). When CMVACHE (Velan et al., 1991*a*) was microinjected into oocytes, active AChE in yields 10–20-fold higher than that observed following RNA injections was obtained (Fig. 1D), demonstrating efficient transcription from this promoter in *Xenopus*.

Transient expression of CMVACHE in *Xenopus* embryos

Microinjected into cleaving *Xenopus* embryos, CMVACHE directed the biosynthesis of rHAChE at levels similar to those observed in DNA-injected oocytes. Yet, the gross morphology and development of CMVACHE-injected embryos appeared completely normal (Fig. 2). Moreover, gross motor function of microinjected embryos, as evaluated by twitching and hatching on day 2, reflexive swimming on day 3, and free swimming on later days, was unimpaired compared with normal, uninjected controls. Microinjected tadpoles survived up to 4 weeks, showing no overt developmental handicaps (data not shown). Following overnight incubation, at which time embryos had reached the late gastrula stage, endogenous AChE levels were negligible, and rHAChE activity represented a 50–100-fold excess over normal (Fig. 3A). From day 2 PF, detectable endogenous AChE activities increased steadily. Using the irreversible AChE inhibitor echothiophate (Neville et al., 1992) to distinguish between endogenous frog AChE and rHAChE (Fig. 3A, inset), we observed the persistence of receding levels of rHAChE for at least 4 days PF. For the first 3 days rHAChE accounted for >50% of the total measured AChE activity in microinjected embryos and resulted in a state of general overexpression com-

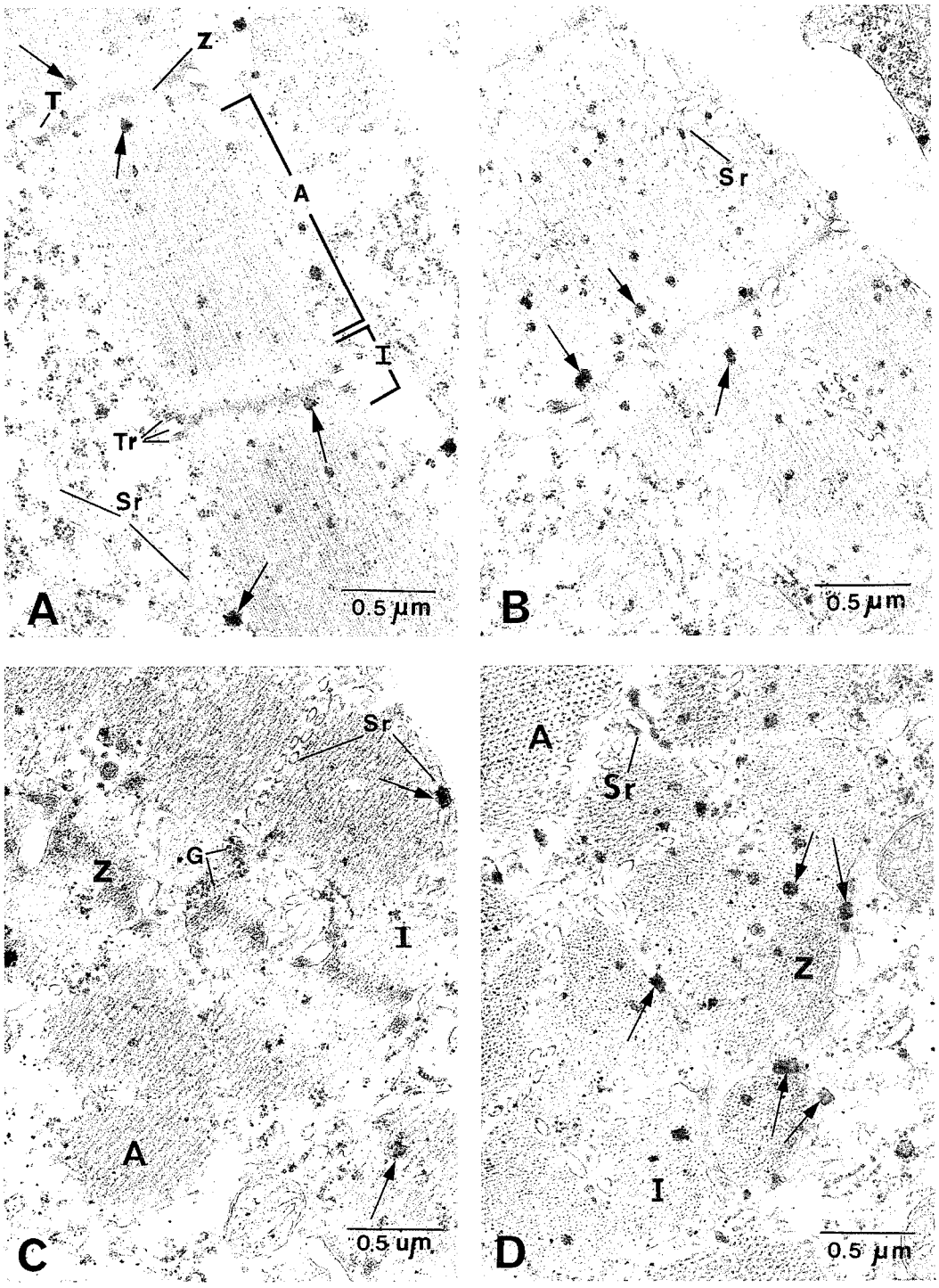

FIG. 5. Disposition of rHAChE in myotomes from 2-day-old microinjected *Xenopus* embryos. Fertilized *Xenopus* eggs were microinjected with 1 ng of CMVACHE, incubated for 2 days at 17°C, fixed, stained, and prepared for electron microscopy as described in Materials and Methods. Uninjected embryos from the same fertilization served as controls and were similarly treated. Arrows mark accumulations of reaction product indicating sites of catalytically active AChE. **A:** Uninjected control myotome in longitudinal section following activity staining for AChE. **B:** Myotome section from CMVACHE-injected embryo. **C:** Uninjected control myotome in transverse section. **D:** Transverse section from CMVACHE-injected embryo. Note the increased intensity of staining in sections from injected embryos versus uninjected controls within the same subcellular compartments, especially within the sarcoplasmic reticulum (Sr). A, A band; I, I band; Z, Z disc; Tr, triad; T, T tubules; G, glycogen particles. Bar = 0.5 μm.

pared with uninjected controls. By day 6 PF, no heterologous enzyme could be detected in homogenates (data not shown). At all time points examined, the level of frog AChE in CMVACHE-injected tadpoles appeared less than that observed in uninjected embryos.

In immunoblot analysis following denaturing gel electrophoresis, rHAChE was observed to comigrate with native human brain AChE, yielding a clearly visible doublet band at ~68 kDa (Fig. 3B). rHAChE was selectively recognized by a pool of mAbs raised against denatured human brain AChE, and no cross-immunoreactivity with embryonic *Xenopus* AChE was observed (Fig. 3B). The doublet band observed may reflect differences in glycosylation (Kronman et al., 1992). Sequential extractions with low-salt, detergent, and high-salt buffers revealed that ~35% of rHAChE synthesized in transiently transgenic embryos was associated with membranes, requiring detergent for solubilization (Table 1). Whereas up to 33% of the endogenous enzyme in day 3 uninjected tadpoles appeared in the high-salt-extractable fraction, salt-soluble rHAChE remained primarily in the low-salt fraction at all days examined (Table 1). Enzyme-antigen immunoassay with a species-specific mAb (mAb 101-1) was used to differentiate between human and frog enzyme in the fractions.

rHAChE remains monomeric in *Xenopus* embryos

To examine the possibility that heterologous human AChE could undergo homomeric oligomeric assembly or interact with either catalytic or noncatalytic subunits of *Xenopus* AChE to produce hybrid oligomers, sucrose density centrifugation and enzyme–antigen immunoassay were performed. At all time points examined, we observed rHAChE exclusively as nonassembled monomers sedimenting at ~3.2S, despite the concomitant accumulation of various multimeric forms of the endogenous frog enzyme (Fig. 4). When oligomeric AChE purified from CMVACHE-transfected cell cultures (Velan et al., 1991*b*) or from human brain (Liao et al., 1992) was preincubated with extracts of day 3 uninjected embryos and similarly analyzed, monomers, dimers, and tetramers were detected, and the distribution of oligomeric forms observed was identical to that in control samples (data not shown). Thus, mAb 101-1 detects all the globular configurations of rHAChE, and proteolytic activity does not appear to degrade stable oligomeric AChE in embryo extracts. Endogenous *Xenopus* AChE appeared primarily as a dimer on day 2 PF with globular tetrameric and asymmetric tailed forms appearing and increasing in content from day 3 onward (Fig. 4, insets). Superposition of the gradients from control and CMVACHE-injected embryos demonstrated that the normal developmental progression of *Xenopus* AChE oligomeric assembly was conserved in CMVACHE-injected embryos despite the high excess of rHAChE monomers (Fig. 4 and data not shown).

Subcellular disposition of rHAChE in myotomes of CMVACHE-injected embryos

Whole-mount cytochemical staining of CMVACHE-injected embryos indicated accumulation of AChE in myotomes 2 days PF (data not shown). We therefore undertook an ultrastructural analysis, at the electron microscope level, of myotomes from 2- and 3-day-old embryos microinjected with CMVACHE as compared with normal uninjected controls. Longitudinal and transverse sections from rostral trunk somites revealed clearly discernible myofibers 2 days PF in both injected and uninjected embryos (Fig. 5). By day 3 PF, both groups displayed significant increases in their numbers of myofibrillar elements and in maturation of the sarcoplasmic reticulum (Fig. 6). To examine the subcellular localization of nascent AChE in transgenic and control embryos, we used cytochemical activity staining (Karnovsky, 1964). In both the experimental and control groups, crystalline deposits of electron-dense reaction product were observed primarily in association with myofibrils, among the myofilaments, and within the sarcoplasmic reticulum (Figs. 5 and 6). Various organelles, including the nuclear membrane, free and bound polyribosomes, Golgi, and sometimes mitochondria, were also stained (Figs. 5 and 6 and data not shown).

At day 2 PF, staining in CMVACHE-injected embryos was conspicuously more pronounced than that observed in uninjected controls, in both the quantity and intensity of reaction product (Fig. 5). However, variability was observed between tissue blocks, probably reflecting mosaic expression of the injected DNA and/or variability in the efficiency of expression between embryos (S.S. and H.S., unpublished data). In longitudinal sections from CMVACHE-injected embryos, staining appeared to be concentrated at the I band of myofibers, particularly around the triad marking the intersection of the sarcoplasmic reticulum and T-tubule systems. In contrast, the sparse staining observed in control sections appeared randomly distributed. By day 3 PF, the general staining intensity in both groups had significantly increased, whereas observable differences between the groups were less dramatic. Cross sections revealed especially prominent staining within the sarcoplasmic reticulum (Fig. 6A and B). Strong staining was now observed at both the A and I bands and, for the first time, within the T-tubules (Fig. 6C and D). Overall, day 2 CMVACHE-injected myotomes resembled day 3 uninjected control myotomes in staining incidence and intensity (Figs. 5A and C and 6B and D).

Ultrastructural consequences of overexpressed AChE in *Xenopus* NMJs

We have previously demonstrated up to 10-fold overexpression of catalytically active AChE in NMJs of CMVACHE-injected embryos 2 days PF (Ben

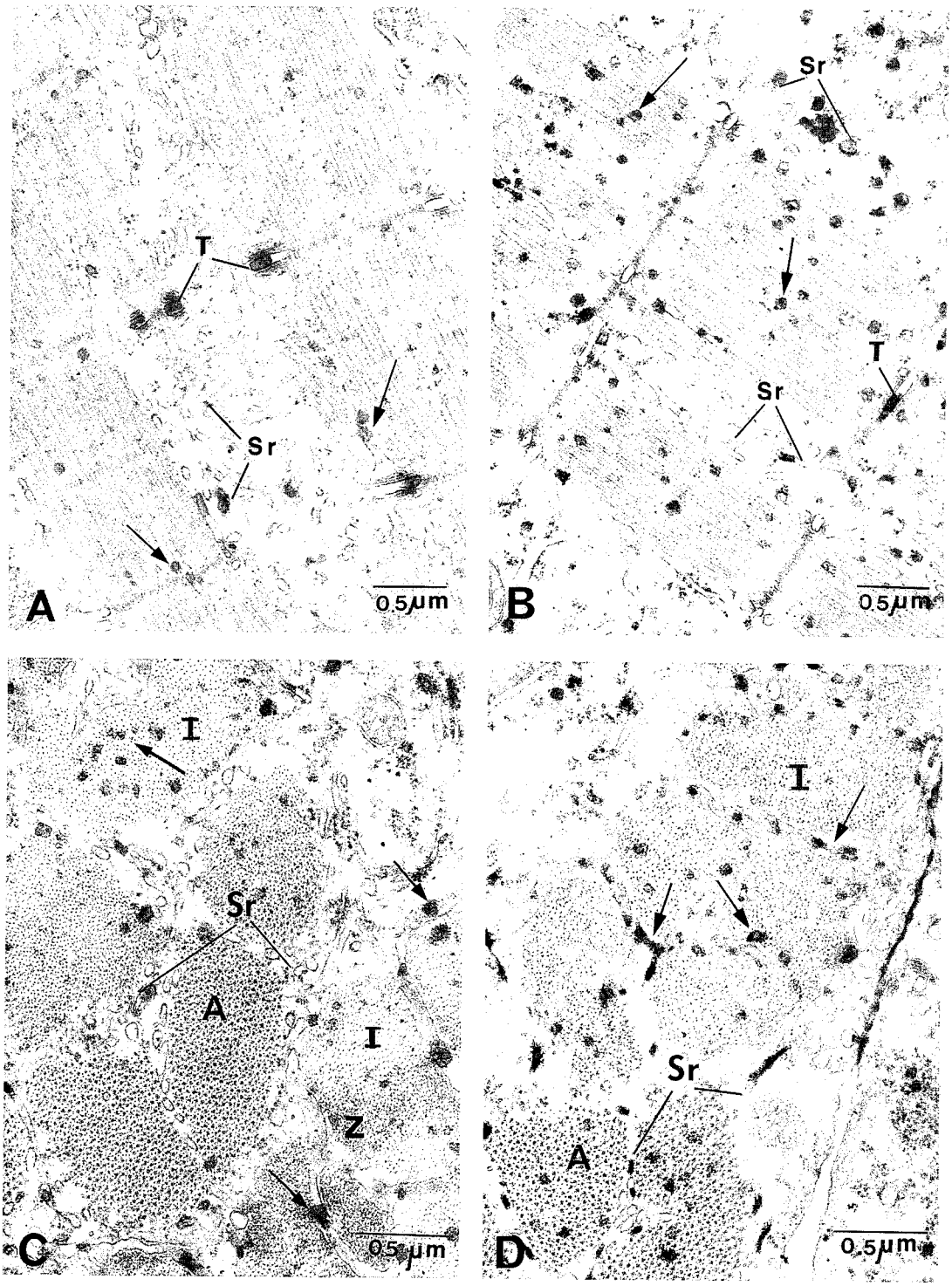

FIG. 6. Overexpression of AChE in myotomes of CMVACHE-injected embryos persists to day 3. Analyses were as in Fig. 5 except that embryos were analyzed after incubation for 3 days. Note the developmental increases in myotomal AChE in both control uninjected (**A** and **C**) and CMVACHE-injected sections (**B** and **D**), especially within the sarcoplasmic reticulum (Sr) and T-tubules (T). Bar = 0.5 μm.

Aziz-Aloya et al., 1993). To examine the persistence of this state and its implications for synaptic ultrastructure, we studied both cytochemically stained and closely appositioned unstained NMJs from 3-day-old injected and control embryos (Fig. 7 and Table 2). In the injected group, 72% of the postsynaptic membrane length (Table 2) was stained, on average, for active AChE. In contrast, only 22% of the postsynaptic length was stained in controls. Moreover, the total area covered by reaction product was approximately

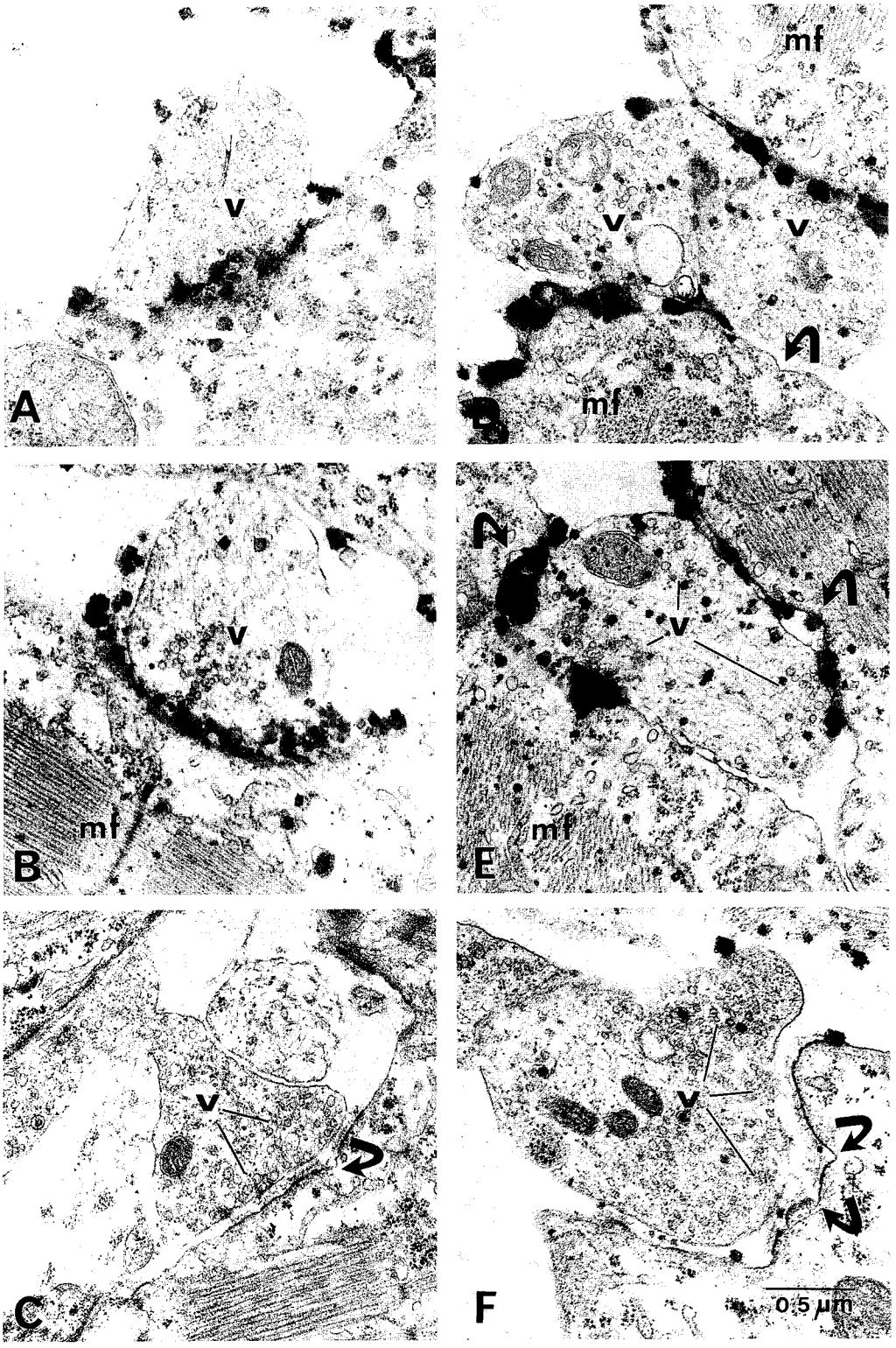

FIG. 7. Structural features in NMJs of 3-day-old CMVACHE-injected *Xenopus* embryos overexpressing AChE. *Xenopus* embryos were cultured for 3 days, fixed, stained for AChE catalytic activity, and examined by transmission electron microscopy as described in Materials and Methods. Two cytochemically stained synapses are presented from uninjected control (**A** and **B**) and CMVACHE-injected (**D** and **E**) embryos. Note the particularly high-density staining in areas directly opposite nerve terminal zones enriched in presynaptic neurotransmitter vesicles (v). **C** and **F:** Representative unstained NMJs from a control and a CMVACHE-injected embryo, respectively. The synapse shown in B represents the highest degree of staining observed in a control section. mf, myofibril. Arrows indicate postsynaptic folds.

TABLE 2. *Overexpression of AChE in NMJs of 3-day-old CMVACHE-injected* Xenopus *embryos*

Experiment	PSL (μm)	SL (μm)	SL/PSL ratio	SA (μm²)
Uninjected	2.57	0.79	0.004	0.156
	3.95	0.79	0.200	0.126
	1.54	0.80	0.060	0.080
	2.35	0.73	0.310	0.082
	1.17	0.44	0.085	0.063
	1.60	0.29	0.180	0.056
	1.02	0.29	0.280	0.040
	0.88	0.58	0.650	0.075
Average ± SD	1.88 ± 0.93	0.58 ± 0.22	0.22 ± 0.19	0.084 ± 0.038
+ CMVACHE	1.76	1.17	0.660	0.284
	2.50	2.05	0.820	0.331
	2.64	1.91	0.720	0.285
	2.50	2.40	0.960	0.476
	3.50	2.03	0.580	0.396
	1.85	1.66	0.900	0.333
	3.10	1.85	0.600	0.535
	3.23	1.76	0.540	0.289
Average ± SD	2.64 ± 0.58	1.85 ± 0.33	0.72 ± 0.15	0.37 ± 0.09
p	<0.01	<0.002	<0.002	<0.002

Eight representative synapses from CMVACHE-injected or control uninjected embryos were assessed for postsynaptic membrane length (PSL), the sum total length covered by reaction product (SL), the fraction of nerve–muscle contact distance displaying reaction product (SL/PSL), and the total stained area (SA). Average ± SD values are given. Measurements were performed on electron micrographs using a hand-held mapping device.

fourfold greater in NMJs from CMVACHE-injected embryos than those from controls (Table 2). In addition, the staining observed in NMJs from injected embryos was considerably more intense than that displayed by control NMJs, forming large black accumulations of reaction product as opposed to the lighter, more diffuse staining observed in controls (Fig. 7A, B, D, and E).

Ultrastructural features of NMJs from injected and uninjected embryos were best discerned in unstained synapses. NMJs from control embryos generally appeared smooth and relatively undeveloped, with up to two secondary folds of the postsynaptic membrane and a single nerve–muscle contact (Fig. 7C). In contrast, NMJs from CMVACHE-injected embryos displayed an average of three secondary folds and one to three discrete contacts between pre- and postsynaptic membranes (Fig. 7F). Furthermore, the average postsynaptic membrane length in NMJs from CMVACHE-injected embryos was 30% larger and considerably less variable than that measured in control embryos (SL; Table 2). Yet, the distance across the synaptic cleft was both larger and more variable in injected embryos than in controls (129 ± 72 vs. 94 ± 23 nm; n = 14). NMJs overexpressing rHAChE thus appeared more developed in their structural buildup than controls.

DISCUSSION

Numerous important nervous system proteins have been expressed in DNA- and RNA-injected oocytes of *X. laevis* (reviewed by Soreq and Seidman, 1992). To study the role and regulation of specific gene products in embryonic development (Vize et al., 1991) and myogenesis (Hopwood et al., 1991), microinjected *Xenopus* embryos have been used. Here, we used microinjected oocytes to demonstrate the efficacy of the CMV promoter in *Xenopus,* observing five- to 10-fold higher levels of heterologous enzyme than that induced by microinjection of in vitro-transcribed mRNA. Although no direct interactions between rHAChE and endogenous *Xenopus* AChE catalytic or structural subunits were observed, calculations of *Xenopus* AChE levels in microinjected embryos indicated that some feedback regulation may be operative in repressing endogenous AChE biosynthesis under conditions of overexpression. Feedback regulation of AChE has been previously demonstrated in chicken retinospheroids (Willbold and Layer, 1992).

Ectopic gene expression/overexpression often results in gross morphogenic aberrations (Harvey and Melton, 1988; McMahon and Moon, 1989; Sokol et al., 1991). Yet, we found that *Xenopus* embryos can tolerate large excesses of catalytically active heterologous AChE without suffering gross morphological or developmental abnormalities. This observation is especially interesting in light of evidence implicating AChE in the early embryonic development of noncholinergic tissues (Drews, 1975) and with developmental processes such as gastrulation and cell migration (Drews, 1975; Fitzpatrick-McElligot and Stent, 1981), nerve outgrowth and differentiation (Layer,

1991), and proliferation and differentiation of hematopoietic cells (Lapidot-Lifson et al., 1989, 1992; Patinkin et al., 1990). As neither the overall rate of development nor general morphology of CMVACHE-injected embryos was altered by 50–100-fold excesses of the active enzyme at the gastrula stage, our findings do not support a role for rHAChE in modulating cell growth, proliferation, or movement in very early *Xenopus* embryogenesis. However, because these biological activities may be unassociated with acetylcholine hydrolysis, they may demonstrate species specificity and remain undetected in our system.

Despite their lack of MyoD elements, some constructs carrying the pan-active CMV promoter (Schmidt et al., 1990) were shown to be expressed in myotomes of transgenic mouse embryos (Kothary et al., 1991). Therefore, the characteristic subcellular segregation of overexpressed rHAChE in muscle may reflect either tissue-specific biosynthesis or posttranslational processing of nascent enzyme present in myotomal progenitor cells at the onset of myogenesis. The high levels of rHAChE present in gastrula-stage embryos may argue for the latter possibility. In that case, the cytochemical data indicate the existence of an intrinsic, evolutionarily conserved property directing the subcellular trafficking of AChE in muscle and thus explain the accumulation of rHAChE in NMJs of ACHE DNA-injected embryos. Furthermore, these results may imply that cotranslational processes are not required for the correct compartmentalization of AChE in muscle cells. In a similar vein, purified recombinant synapsin was shown to be incorporated into synaptic nerve terminals of cultured myotomes following microinjection into fertilized *Xenopus* eggs (Lu et al., 1992).

The general state of myotomal overexpression induced by microinjection of CMVACHE persisted at least 3 days. The area covered by reaction product in cytochemically stained NMJs from day 3, CMVACHE-injected embryos was four- to fivefold over that observed in controls. This figure represents a twofold lower excess than that measured in day 2 NMJs (Ben Aziz-Aloya et al., 1993) yet is slightly greater than the ratio of rHAChE to frog AChE as determined in homogenates at day 3 (Fig. 3A). This apparent reduction in the level of synaptic overexpression from day 2 to day 3 PF may reflect the overall decline in total rHAChE activity observed during this period. However, because this calculation does not consider the higher-density staining observed in NMJs from CMVACHE-injected embryos, it represents an underestimate of the actual synaptic AChE content. Therefore, our data indicate enhanced stability of rHAChE at the NMJ compared with the total pool, a conclusion consistent with the observation that extracellular matrix-associated AChE persists in situ long after denervation of adult frog skeletal muscle (Anglister and McMahan, 1985).

Mammalian cells cotransfected with cDNAs encoding catalytic and noncatalytic AChE subunits (Krejci et al., 1991) produce multimeric globular and asymmetric AChEs, indicating that spatial coexistence may normally be the only requirement for multimeric assembly. Human cell lines transfected with various CMVACHE constructs similarly express and secrete homooligomers (Velan et al., 1991*a*; Kronman et al., 1992). rHAChE displayed oligomeric assembly in microinjected *Xenopus* oocytes but not in developing embryos, where only monomeric rHAChE was detected. Nonetheless, rHAChE was found to accumulate in its natural subcellular compartments and was correctly transported to the NMJ of transiently transfected tadpoles. These findings are puzzling in light of the demonstration that secretion appears linked to oligomerization in transfected human 293 cells (Velan et al., 1991*b*; Kerem et al., 1993). Furthermore, the lack of demonstrable oligomeric assembly leaves the mode of association of rHAChE with the extracellular surface unexplained. It is noteworthy, however, that DNA constructs encoding the parallel AChE form from *Torpedo* (Duval et al., 1992) and the rat (Legay et al., 1993) also gave rise to globular amphiphilic AChE forms in transfected mammalian cells, including type II amphiphilic monomers.

In humans, ultrastructural and physiological alterations of the NMJ have been associated with congenital AChE and AChR deficiencies (Wokke et al., 1989; Jennekens et al., 1992) and may be associated with changes in the balance between these two molecules at the synapse. In one of these syndromes, patients presented, in addition to AChE/AChR deficits, NMJs displaying decreased miniature end-plate potentials, reduced postsynaptic membrane lengths, and severely impaired postsynaptic secondary folding (Smit et al., 1988)—opposite features to those observed in our NMJs overexpressing AChE. It is yet unclear whether the reduced expression of synaptic AChE and/or AChR represents a cause or an outcome of the ultrastructural aberrations observed in these patients. Our current observations suggest that disturbed regulation of AChE may, indeed, carry ultrastructural consequences for synaptic development. It will thus be interesting to assess the impact of AChE overexpression on the expression and organization of AChR and other key synaptic proteins in these transiently transgenic tadpoles.

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REFERENCES

Anglister L. and McMahan U. J. (1985) Basal lamina directs acetylcholinesterase accumulation at synaptic sites in regenerating muscle. *J. Cell Biol.* **101,** 735–743.

Ben Aziz-Aloya R., Seidman S., Timberg R., Sternfeld M., Zakut H., and Soreq H. (1993) Expression of a human acetylcholinesterase promoter–reporter construct in developing neuromuscular junctions of *Xenopus* embryos. *Proc. Natl. Acad. Sci. USA* **90,** 2471–2475.

Brandan E., Maldonado M., Garrido J., and Inestrosa N. C. (1985) Anchorage of collagen-tailed acetylcholinesterase is mediated by heparan sulfate proteoglycans. *J. Cell Biol.* **101,** 985–992.

Brodbeck U. and Liao J. (1992) Subunit assembly and glycosylation of acetylcholinesterase from mammalian brain, in *Multidisciplinary Approaches to Cholinesterase Functions* (Shafferman A. and Velan B., eds), pp. 33–38. Plenum Press, New York.

Changeux J. P. (1991) Compartmentalized transcription of acetylcholine receptor genes during motor endplate epigenesis. *New Biol.* **3,** 413–429.

Cohen M. W. (1980) Development of an amphibian neuromuscular junction *in vivo* and in culture. *J. Exp. Biol.* **89,** 43–56.

Deuchar E. M. (1966) *Biochemical Aspects of Amphibian Development.* Methuen and Co. Ltd., London.

Drews U. (1975) Cholinesterase in embryonic development. *Prog. Histochem. Cytochem.* **7,** 1–52.

Duval N., Massoulie J., and Bon S. (1992) H and T subunits of acetylcholinesterase from *Torpedo*, expressed in COS cells, generate all types of molecular forms. *J. Cell Biol.* **118,** 641–653.

Fitzpatrick-McElligot S. and Stent G. S. (1981) Appearance and localization of acetylcholinesterase in embryos of the leech *Helodbella triserialis*. *J. Neurosci.* **1,** 901–907.

Flucher B. E. and Daniels M. D. (1989) Distribution of Na^+ channels and ankyrin in neuromuscular junctions is complementary to that of acetylcholine receptors and the 43 Kd protein. *Neuron* **3,** 163–175.

Froehner S. C. (1991) The submembrane machinery for nicotinic acetylcholine receptor clustering. *J. Cell Biol.* **114,** 1–7.

Gennari K. and Brodbeck U. (1985) Molecular forms of acetylcholinesterase from human caudate nucleus: comparison of salt-soluble and detergent-soluble tetrameric enzyme species. *J. Neurochem.* **44,** 697–704.

Gindi T. and Knowland J. (1979) The activity of cholinesterases during the development of *Xenopus laevis*. *J. Embryol. Exp. Morphol.* **51,** 209–215.

Hall Z. W. (1973) Multiple forms of acetylcholinesterase and their distribution in endplate and non-endplate regions of rat diaphragm muscle. *J. Neurobiol.* **4,** 343–361.

Harvey R. P. and Melton D. A. (1988) Microinjection of synthetic Xhox-1A homeobox mRNA disrupts somite formation in developing *Xenopus* embryos. *Cell* **53,** 687–697.

Hopwood N. D., Pluck A., and Gurdon J. B. (1991) *Xenopus* Myf-5 marks early muscle cells and can activate muscle genes ectopically. *Development* **111,** 551–560.

Jennekens F. G. I., Hesselmans L. F. G. M., Veldman H., Jansen E. N. H., Spaans F., and Molenaar P. C. (1992) Deficiency of acetylcholine receptors in a case of end-plate acetylcholinesterase deficiency: a histochemical investigation. *Muscle Nerve* **15,** 63–72.

Karnovsky M. J. (1964) The localization of cholinesterase activity in rat cardiac muscle by electron microscope. *J. Cell Biol.* **23,** 217–232.

Kerem A., Kronman C., Bar-Nun S., Shafferman A., and Velan B. (1993) Interaction between assembly and secretion of recombinant human acetylcholinesterase. *J. Biol. Chem.* **268,** 180–184.

Kothary R., Barton S. C., Franz T., Norris M. L., Hettle S., and Surani A. M. H. (1991) Unusual cell specific expression of a major human cytomegalovirus immediate early gene promoter–lacZ hybrid gene in transgenic mouse embryos. *Mech. Dev.* **35,** 25–31.

Krejci E., Coussen F., Duval N., Chatel J.-M., Legay C., Puype M., Vandekerckhove J., Cartaud J., Bon S., and Massoulie J. (1991) Primary structure of a collagenic tail peptide of *Torpedo* acetylcholinesterase: co-expression with catalytic subunit induces the production of collagen-tailed forms in transfected cells. *EMBO J.* **10,** 1285–1293.

Kronman C., Velan B., Gozes Y., Leitner M., Flashner Y., Lazar A., Marcus D., Sery T., Papier A., Grosfeld H., Cohen S., and Shafferman A. (1992) Production and secretion of high levels of recombinant human acetylcholinesterase in cultured cell lines: microheterogeneity of the catalytic subunit. *Gene* **121,** 295–304.

Kullberg R. W., Lentz T. L., and Cohen M. W. (1977) Development of the myotomal neuromuscular junction in *Xenopus laevis:* an electrophysiological and fine-structural study. *Dev. Biol.* **60,** 101–129.

Kullberg R. W., Mikelberg F. S., and Cohen M. W. (1980) Contribution of cholinesterase to developmental decreases in the time course of synaptic potentials at an amphibian neuromuscular junction. *Dev. Biol.* **75,** 255–267.

Lapidot-Lifson Y., Prody C. A., Ginzberg D., Meytes D., Zakut H., and Soreq H. (1989) Co-amplification of human acetylcholinesterase and butyrylcholinesterase in blood cells: correlation with various leukemias and abnormal megakaryocytopoiesis. *Proc. Natl. Acad. Sci. USA* **86,** 4715–4717.

Lapidot-Lifson Y., Patinkin D., Prody C., Ehrlich G., Seidman S., Ben-Aziz R., Eckstein F., Benseler F., Zakut H., and Soreq H. (1992) Cloning and antisense oligodeoxynucleotide inhibition of a human homolog of cdc2 required in hematopoiesis. *Proc. Natl. Acad. Sci. USA* **89,** 579–583.

Layer P. (1991) Cholinesterases during avian development. *Cell. Mol. Neurobiol.* **11,** 7–34.

Legay C., Bon S., Vernier P., Coussen F., and Massoulié J. (1993) Cloning and expression of a rat acetylcholinesterase subunit: generation of multiple molecular forms, complementarity with a *Torpedo* collagenic subunit. *J. Neurochem.* **60,** 337–346.

Liao J., Heider H., Sun M.-C., and Brodbeck U. (1992) Different glycosylation in acetylcholinesterases from mammalian brain and erythrocytes. *J. Neurochem.* **58,** 1230–1238.

Lu B., Greengard P., and Poo M. (1992) Exogenous synapsin I promotes functional maturation of developing neuromuscular synapses. *Neuron* **8,** 521–529.

McMahan U. J. (1990) The agrin hypothesis. *Cold Spring Harb. Symp. Quant. Biol.* **55,** 407–418.

McMahon A. P. and Moon R. T. (1989) Ectopic expression of the proto-oncogene int-1 in *Xenopus* embryos leads to duplication of the embryonic axis. *Cell* **58,** 1075–1084.

Neville L. F., Gnatt A., Loewenstein Y., Seidman S., Ehrlich G., and Soreq H. (1992) Intra-molecular relationships in cholinesterases revealed by oocyte expression of site-directed and natural variants of human BCHE. *EMBO J.* **11,** 1641–1649.

Ohlendieck K., Ervasti J. M., Matsumura K., Kahl S. D., Levelille C. J., and Campbell K. P. (1991) Dystrophin-related protein is localized to neuromuscular junctions of adult skeletal muscle. *Neuron* **7,** 499–508.

Patinkin D., Seidman S., Eckstein F., Benseler F., Zakut H., and Soreq H. (1990) Manipulations of cholinesterase gene expression modulate murine megakaryocytopoiesis *in vitro*. *Mol. Cell. Biol.* **10,** 6046–6050.

Pavlath G. K., Rich K., Webster S. G., and Blau H. M. (1989) Localization of muscle gene products in nuclear domains. *Nature* **337,** 570–573.

Phillips W. D., Kopta C., Blount P., Gardner P. D., Steinbach J. H., and Merlie J. P. (1991) ACh receptor-rich membrane domains organized in fibroblasts by recombinant 43 kilodalton protein. *Nature* **251,** 568–570.

Ralston E. and Hall Z. W. (1989) Transfer of a protein encoded by a single nucleus to nearby nuclei in multinucleated myotubes. *Nature* **244,** 1066–1069.

Rossi S. G. and Rotundo R. L. (1992) Cell surface acetylcholinesterase molecules on multinucleated myotubes are clustered over the nucleus of origin. *J. Cell Biol.* **119,** 1657–1667.

Rotundo R. L. (1990) Nucleus-specific translation and assembly of

acetylcholinesterase in multinucleated muscle cells. *J. Cell Biol.* **110,** 715–719.

Schmidt E. V., Christoph G., Zeller R., and Leder P. (1990) The cytomegalovirus enhancer: a Pan-active control element in transgenic mice. *Mol. Cell. Biol.* **10,** 4406–4411.

Smit L. M. E., Hageman G., Veldman H., Molenaar P. C., Oen B. S., and Jennekens F. G. I. (1988) A myasthenic syndrome with congenital paucity of secondary clefts: CPSC syndrome. *Muscle Nerve* **11,** 337–348.

Sokol S., Christian J. L., Moon R. T., and Melton D. A. (1991) Injected Wnt RNA induces a complete body axis in *Xenopus* embryos. *Cell* **67,** 741–752.

Soreq H. and Seidman S. (1992) *Xenopus* oocyte microinjection: from gene to protein, in *Methods in Enzymology* (B. Rudy and L. Iversen, eds), pp. 225–265. Academic Press, San Diego.

Soreq H., Seidman S., Dreyfus P. A., Zevin-Sonkin D., and Zakut H. (1989) Expression and tissue specific assembly of cloned human butyrylcholine esterase in microinjected *Xenopus laevis* oocytes. *J. Biol. Chem.* **264,** 10608–10613.

Soreq H., Ben-Aziz R., Prody C., Seidman S., Gnatt A., Neville L., Lieman-Hurwitz J., Lev-Lehman E., Ginzberg D., Lapidot-Lifson Y., and Zakut H. (1990) Molecular cloning and construction of the coding region for human acetylcholinesterase reveals a G,C rich attenuating structure. *Proc. Natl. Acad. Sci. USA* **87,** 9688–9692.

Velan B., Kronman C., Grosfeld H., Leitner M., Gozes Y., Flashner Y., Sery T., Cohen S., Ben-Aziz R., Seidman S., Shafferman A., and Soreq H. (1991*a*) Recombinant human acetylcholinesterase is secreted from transiently transfected 293 cells as a soluble globular enzyme. *Cell. Mol. Neurobiol.* **11,** 143–156.

Velan B., Grosfeld H., Kronman C., Leitner M., Gozes Y., Lazar A., Flashner Y., Marcus D., Cohen S., and Shafferman A. (1991*b*) The effect of elimination of intersubunit disulfide bonds on the activity, assembly, and secretion of recombinant human acetylcholinesterase. *J. Biol. Chem.* **266,** 23977–23984.

Vize P. D., Melton D. A., Hemmati-Brivanlou A., and Harland R. M. (1991) Assays for gene function in developing *Xenopus* embryos, in *Methods in Cell Biology* (Kay B. K. and Peng H. B., eds), pp. 367–387. Academic Press, San Diego.

Wallace B. G. (1991) The mechanisms of agrin-induced acetylcholine receptor aggregation. *Philos. Trans. R. Soc. Lond. Biol.* **331,** 272–280.

Willbold E. and Layer P. (1992) Formation of neuroblastic layers in chicken retinospheroids: the fibre layer of Chievitz secludes AChE positive cells from mitotic cells. *Cell Tissue Res.* **268,** 401–408.

Wokke J. H. J., Jennekens F. G. I., Molenaar P. C., Van den Oord C. J. M., Oen B. S., and Busch H. F. M. (1989) Congenital paucity of secondary synaptic clefts (CPSC) syndrome in 2 adult siblings. *Neurology* **38,** 648–654.

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Synaptic and Epidermal Accumulations of Human Acetylcholinesterase Are Encoded by Alternative 3′-Terminal Exons

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Tissue-specific heterogeneity among mammalian acetylcholinesterases (AChE) has been associated with 3′ alternative splicing of the primary AChE gene transcript. We have previously demonstrated that human AChE DNA encoding the brain and muscle AChE form and bearing the 3′ exon E6 (ACHE-E6) induces accumulation of catalytically active AChE in myotomes and neuromuscular junctions (NMJs) of 2- and 3-day-old *Xenopus* embryos. Here, we explore the possibility that the 3′-terminal exons of two alternative human AChE cDNA constructs include evolutionarily conserved tissue-recognizable elements. To this end, DNAs encoding alternative human AChE mRNAs were microinjected into cleaving embryos of *Xenopus laevis*. In contrast to the myotomal expression demonstrated by ACHE-E6, DNA carrying intron I4 and alternative exon E5 (ACHE-I4/E5) promoted punctuated staining of epidermal cells and secretion of AChE into the external medium. Moreover, ACHE-E6-injected embryos displayed enhanced NMJ development, whereas ACHE-I4/E5-derived enzyme was conspicuously absent from muscles and NMJs and its expression in embryos had no apparent effect on NMJ development. In addition, cell-associated AChE from embryos injected with ACHE-I4/E5 DNA was biochemically distinct from that encoded by the muscle-expressible ACHE-E6, displaying higher electrophoretic mobility and greater solubility in low-salt buffer. These findings suggest that alternative 3′-terminal exons dictate tissue-specific accumulation and a particular biological role(s) of AChE, associate the 3′ exon E6 with NMJ development, and indicate the existence of a putative secretory AChE form derived from the alternative I4/E5 AChE mRNA.

Acetylcholinesterase (AChE) is accumulated at neuromuscular junctions (NMJs) (25), where it serves a vital function in modulating cholinergic neurotransmission (reviewed in reference 31). The molecular mechanisms by which AChE and other synaptic proteins accumulate in the NMJ are poorly understood. Compartmentalized transcription and translation in and around the junctional nuclei probably contribute to the NMJ localization of AChE (9). However, the high concentration of AChE at NMJs suggests that an additional step(s) may be required to actively accumulate this molecule in its ultimate synaptic destination. In that case, it is possible to postulate the existence of a unique molecular tag identifying some AChE forms as NMJ bound. We have previously offered evidence that an evolutionarily conserved NMJ-recognizable signal is embedded within the primary amino acid sequence of the major brain and muscle (brain-muscle) form of AChE (1). In the present report, we trace this signal as derived from an alternative 3′ exon in the human AChE gene.

In addition to its synaptic location, AChE is known to exist in several nonneuronal cell types, including epidermal and hematopoietic cells (31). Recent studies have correlated this particular tissue distribution of AChE with the 3′ alternative splicing patterns of the mRNA transcripts encoding the AChE protein. The dominant brain-muscle AChE form found in NMJs (AChE-T) was shown to be encoded by an mRNA carrying exon E1 and the invariant coding exons E2, E3, and E4, spliced to alternative exon E6 (1, 15). AChE mRNA bearing exons E1 through E4 and alternative exon E5 encodes the glycolipid phosphatidylinositol (GPI)-linked form of AChE characteristic of vertebrate erythrocytes (AChE-H) (13, 16). An additional readthrough mRNA species retaining the intronic sequence I4 located immediately 3′ to exon E4 was previously observed in rodent bone marrow and erythroleukemic cells (13, 16) and in various tumor cell lines of human origin (11). The tissue-specific posttranscriptional and posttranslational management of AChE mRNA and its polypeptide products raised the question of whether alternative 3′ exons and/or C-terminal peptides play a role in mediating the accumulation of AChE in NMJs as opposed to other tissues expressing this enzyme.

To examine the molecular mechanisms underlying the tissue-specific accumulation patterns of AChE, we established an in vivo model for the expression of alternative human AChEs in transiently transgenic embryos of *Xenopus laevis*. Placed downstream of the cytomegalovirus (CMV) promoter-enhancer unit (32) and microinjected into fertilized eggs of *X. laevis*, DNA carrying the invariant coding exons and alternative exon E6 (ACHE-E6) directed the production of catalytically active AChE which accumulated in muscle cells, nerve terminals, and NMJs of 2- and 3-day-old embryos (1, 27). Here, we have constructed an additional CMV ACHE plasmid (ACHE-I4/E5), potentially encoding the remaining two alternative AChE mRNAs, and compared its expression pattern in microinjected *Xenopus* embryos with that of ACHE-E6. Our findings indicate that tissue-specific accumulation of AChE may be dictated by alternative splicing of AChE mRNAs, that the 3′-terminal exon E6 plays an essential role in accumulation of AChE in NMJs, where it enhances NMJ development, and that the readthrough AChE mRNA may engender a unique secre-

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tory form of human AChE accumulated in epithelial cells, which is excluded from NMJs.

MATERIALS AND METHODS

Vectors. The plasmid referred to here as ACHE-E6 and employed to express the major brain-muscle form of AChE has been described in detail (CMV ACHE) (32). This plasmid contains the AChE-encoding exons E2, E3, E4, and E6 (30) downstream of the CMV promoter and is followed by the simian virus 40 polyadenylation site. ACHE-E6 was used to construct ACHE-I4/E5 by exchanging the cDNA restriction fragment *Not*I-*Hpa*I with the genomic fragment *Not*I-*Hin*dII (see Fig. 1). ACHE-I4/E5 potentially encodes both the GPI-linked erythrocyte AChE form generated by splicing of I4 and/or the readthrough AChE form (11). Repeated DNA sequencing of this plasmid DNA, using a Hot Tub PCR cycle sequencing kit (Amersham, Amersham, United Kingdom) confirmed that the I4 domain includes a stop codon, correcting previous erroneous sequencing (11).

***Xenopus* embryo microinjections.** In vitro fertilization and microinjection of mature *Xenopus* eggs were performed as described elsewhere (27), except that embryos were raised at 19 to 21°C.

RT-PCR procedure and primers. Total RNA was extracted from *Xenopus* embryos 1 day after injection by the RNAsol-B method (Cinna/Biotecx), according to the protocol supplied by the manufacturer, and was treated with DNase (Promega) as previously described (1). Reverse transcription followed by PCR (RT-PCR) analyses were performed as detailed elsewhere (11), using a Perkin-Elmer Cetus thermal controller. Amplification was for 1 min at 94°C [first cycle, 3 min], 1 min at 65°C, and 1 min at 72°C [last cycle, 6 min], performed with the following primer pairs: pair 1, E3/1522+ (5′-CGGGTCTACGCCTACGTCTTT GAACACCGTGCTTC-3′) and E6/2003− (5′-CACAGGTCTGAGCAGCGAT CCTGCTTGCTG-3′); pair 2, E3/1522+ and E5/1917− (5′-ATGGGTGAAGC CTGGGCAGGTG-3′); pair 3, E3/1522+ and I4/1939− (5′-GGTTACACTGGC GGGCTCC-3′); pair 4, E3/1522+ and E4/1797− (5′-CAGGTCCAGACTAAC GTACTGCTGAGCCCCCGCCG-3′). The primers were named according to their position in the human AChE alternative coding sequences (11, 30) and designated upstream (+) or downstream (−) per their orientation. Amplification products (20%) were electrophoresed on a 2% agarose gel and UV photographed (320 nm).

AChE activity assays and subcellular fractionations. AChE activities were determined with a standard colorimetric assay adapted to a 96-well microtiter plate (20, 27). Assays were performed in 0.1 M phosphate buffer (pH 7.4)–0.5 mM dithio-bis-nitrobenzoic acid–1 mM acetylthiocholine substrate at room temperature. Optical density at 405 nm was monitored for 20 min at 3- to 5-min intervals.

Subcellular fractionation of 1-day-old embryos into low-salt (0.01 M Tris-HCl [pH 7.4], 0.05 M $MgCl_2$, 144 mM NaCl), low-salt–detergent (1% Triton X-100 in 0.01 M Na phosphate [pH 7.4]), and high-salt (1 M NaCl in 0.01 M Na phosphate [pH 7.4]) buffers was performed as previously described (27).

Nondenaturing gel electrophoresis. Electrophoresis was performed in 6% polyacrylamide gels (32). Wherever noted, 0.5% Triton X-100 was included. The gels were run in the cold for 2 to 4 h, rinsed two to three times with double-distilled water, and stained for several hours or overnight for catalytically active AChE by the thiocholine method for histochemical staining of AChE developed by Karnovsky and Roots (10) and detailed below.

Whole-mount cytochemical staining. Two-day-old *Xenopus* embryos were fixed for 20 min in 4% paraformaldehyde (in 0.6× phosphate-buffered saline [PBS]), rinsed three times with PBS, and transferred to a clean glass vial. Fixed embryos were incubated in staining solution (0.67 mM acetylthiocholine, 5 mM sodium citrate, 3 mM cupric sulfate, 0.5 mM potassium ferricyanide in 0.1 M acetate buffer [pH 5.9]) overnight at room temperature with gentle rotation, rinsed several times with PBS, and postfixed with 2.5% glutaraldehyde for 1 h. The embryos were then dehydrated by successive transfers through 30, 50, 70, and 100% methanol, mounted in Murray's clearing solution (benzyl alcohol-benzyl benzoate, 1:2), and viewed under low magnification with a Zeiss stereomicroscope. Clearing permitted visualization of internal structures and improved with time up to 18 to 24 h.

Electron microscopy and morphometric analyses. Histochemical staining and transmission electron microscopy were performed as previously described in detail (1, 27). Morphometric analyses of NMJs from 2-day-old embryos raised at 21°C were performed with the SigmaScan software (Jandel Co., Berkeley, Calif.) and an IBM-compatible personal computer.

RESULTS

Alternative mRNAs dictate specific accumulation of AChE in muscle or epidermis. To examine the ability of alternative splicing to account for tissue-specific accretion of AChE, in vitro-fertilized *Xenopus* eggs were microinjected with 1 ng of ACHE-E6 or ACHE-I4/E5 DNA (Fig. 1). The resultant embryos were raised for 2 to 3 days, fixed, and stained for catalytically active enzyme. Following injection of ACHE-E6, encoding the brain-muscle form of AChE, 2-day-old tailbud embryos displayed conspicuous overexpression of AChE in the developing myotomes (Fig. 2, top panels). Myotomal overexpression was primarily observed as pronounced longitudinal staining along the plane of the muscle fibers between the vertical bands representing natural intersomitic accumulations of AChE. With the exception of brain tissues, no other tissues displayed consistently or prominently enhanced staining. However, myotomal overexpression of AChE was clearly mosaic, varying in intensity within and between individual somites. Uninjected control embryos displayed the characteristic transverse staining along the junctions between somites but only faint staining within the myotomes (Fig. 2, top panels).

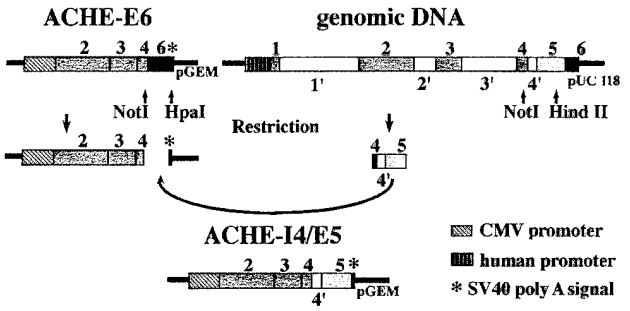

FIG. 1. DNA constructs. The DNA constructs employed in this study are presented. See Materials and Methods for details. SV40, simian virus 40.

In contrast to the striking accumulation of ACHE-E6-derived AChE in myotomes, we did not observe any enhancement of staining in myotomes of embryos injected with ACHE-I4/E5. Rather, we noted pronounced punctuated staining of the epidermis which was never seen in uninjected embryos (Fig. 2, bottom panels). These differential staining patterns were probably not due to variable levels of AChE mRNA, as whole-mount in situ hybridization (9) revealed similar ubiquitous distributions of both transcripts (data not shown). Epidermal staining could be observed over the entire body along both the rostral-caudal and dorsal-ventral axes. Intersomitic staining was unaffected by overexpression of ACHE-I4/E5. Although limited epidermal staining was occasionally observed in ACHE-E6-injected embryos, this phenomenon appeared restricted to sites of particularly high myotomal expression and was considerably less well defined (Fig. 2, bottom panels). These observations indicated that AChE derived from ACHE-E6 DNA was specifically accumulated in muscle, whereas AChE derived from ACHE-I4/E5 was accumulated in the epidermis.

ACHE-I4/E5 is excluded from the NMJ localization characteristic of ACHE-E6 and plays no role in NMJ development. Electron microscopic analysis of myotomes stained for catalytically active AChE revealed conspicuous overexpression of enzyme in NMJs of ACHE-E6-injected embryos and correlated overexpression with alterations in synaptic ultrastructure (27). To finely examine the potential for ACHE-I4/E5-derived AChE to be similarly localized, we performed morphometric analyses of cross sections from a series of stained NMJs from DNA-injected or control uninjected embryos (Fig. 3A through C). The average AChE-stained cross-sectional area of NMJs from ACHE-E6-injected embryos reached three or four times that observed in NMJs from uninjected embryos (0.33 ± 0.29 μm^2 versus 0.08 ± 0.09 μm^2). In contrast, the average stained area of NMJs from embryos injected with ACHE-I4/E5 (0.14 ± 0.03 μm^2) displayed only a minor increase compared with

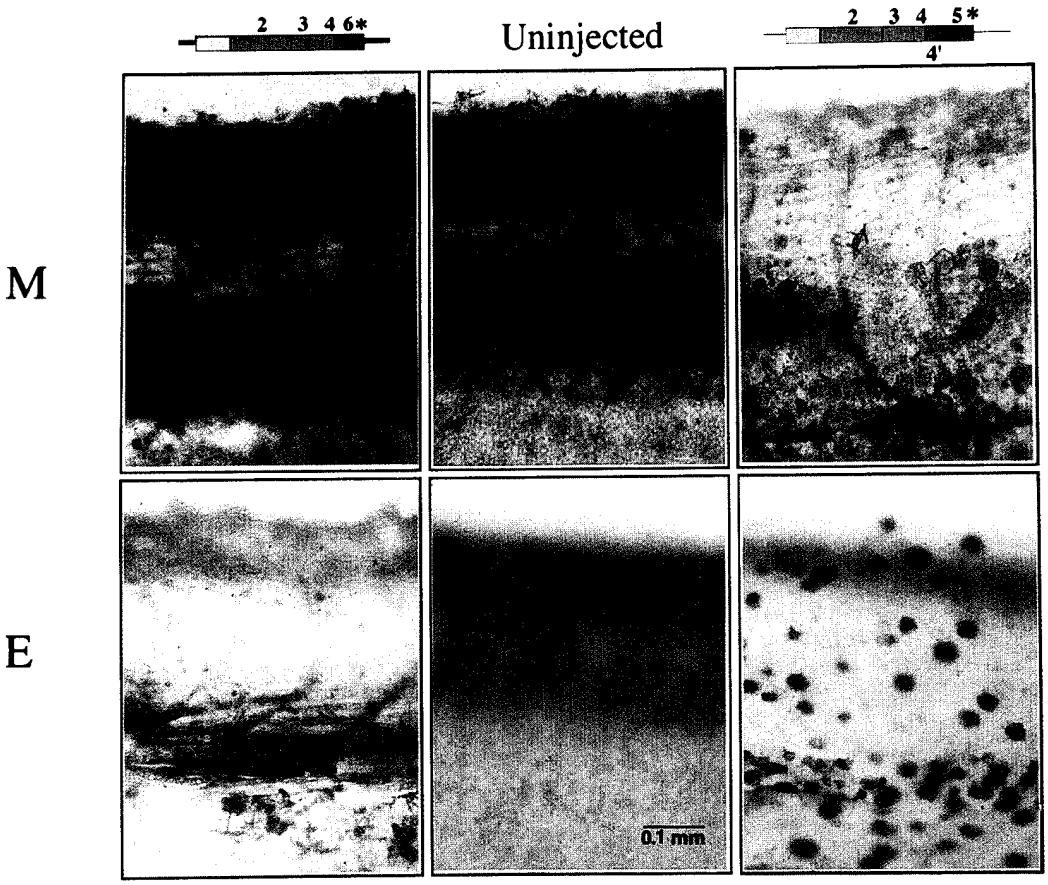

FIG. 2. Alternative AChE mRNAs dictate tissue-specific accumulation of AChE. DNA-injected or control uninjected *Xenopus* embryos were raised for 2 days at 19°C, fixed, stained for catalytically active AChE, and examined in whole mount as described in Materials and Methods. Schematic representations of the microinjected DNAs which gave rise to the staining pattern exhibited in each micrograph are shown (see Fig. 1). (M) Micrographs taken at the inner focal depth of muscle; (E) micrographs taken at the higher focal depth of epidermis. (M [left]) Midtrunk myotomes from ACHE-I4/E5-injected embryo. Note, in addition to the natural intersomitic vertical bands of brown stain indicating sites of AChE activity, dark—almost black—horizontal bands of staining parallel to the plane of the myotomal muscle fibers. This type of longitudinal staining, although variable in intensity along the body and between embryos, was observed exclusively in ACHE-E6-expressing embryos and served as an unequivocal marker for overexpression from this plasmid. (M [center]) Midbody view of uninjected control embryo. Note the accumulation of reaction product at the junctions flanking the individual somites and the very light staining along the horizontal plane of the muscle fibers. (M [right]) Faintly stained myotomes from ACHE-I4/E5-injected embryo. Note that staining is considerably lighter than in control, uninjected embryos. (E [left]) Unlabeled epidermis of ACHE-E6-injected embryo. When photographed at the higher focal depth of the epidermis, ACHE-E6 embryos displayed faint reflections of the deeper muscle stain whereas no stainable enzyme could be detected in epidermis. (E [center]) Unlabeled epidermis of control embryo. Focusing on epidermis, uninjected control embryos revealed no epidermal stain. Faint staining of myotomes below could be observed. (E [right]) ACHE-I4/E5-expressing embryo displaying punctuated epidermal staining. The plane of focus is slightly higher than that in the upper panels. Note the irregular spotted appearance of the skin resulting from ACHE-I4/E5 overexpression and the complete absence of enhanced staining in the myotomes.

that for the controls. Transverse sections of myofibers displayed a parallel pattern of highly intensified staining in myotomes of ACHE-E6-injected embryos compared with both ACHE-I4/E5-injected and uninjected controls (Fig. 3D through F). A twofold increase in mean postsynaptic membrane length was associated with ACHE-E6-mediated overexpression compared with that for the controls (3.8 ± 2.1 μm versus 2.2 ± 1.3 μm). In contrast, the average postsynaptic length observed in ACHE-I4/E5-injected embryos (1.9 ± 0.2 μm) was approximately the same as that for the controls.

At least 40% of NMJs from uninjected and ACHE-I4/E5-injected embryos displayed postsynaptic lengths less than 3 μm. In contrast, these small synapses were not observed in ACHE-E6-injected embryos (Fig. 4). Rather, a class of large NMJs (>4 μm) rarely observed in control or ACHE-I4/E5-injected embryos dominated in those transgenic for ACHE-E6. When the AChE-stained area was calculated as a function of postsynaptic length, ACHE-I4/E5-injected and control uninjected embryos displayed a similar, constant relationship between these parameters, whereas ACHE-E6-injected embryos displayed a higher ratio for all length categories (Fig. 4). Thus, there appeared to be a causal correlation between AChE overexpression in muscle and enhanced postsynaptic length in individual synapses, confirmed by the lack of effect by ACHE-I4/E5. Together, these observations imply a specific role for the E6 exon in localizing AChE to NMJs and demonstrate that the effects of AChE overexpression on NMJ biogenesis are dependent on the muscle localization of the enzyme.

The I4/E5 domain directs AChE accumulation in ciliated epidermal cells and its excretion. To study the cellular and subcellular distribution of overexpressed AChE in epidermal cells from embryos injected with ACHE-I4/E5, we examined epidermis from cytochemically stained embryos by electron microscopy. Two types of cells were labelled by the electron-dense crystals of the AChE reaction product: (i) ciliated cells derived from the inner, sensorial, epidermal layer and (ii) mucus-discharging secretory cells (Fig. 5) (2). There were many fewer ciliated cells than secretory cells. Yet, the inci-

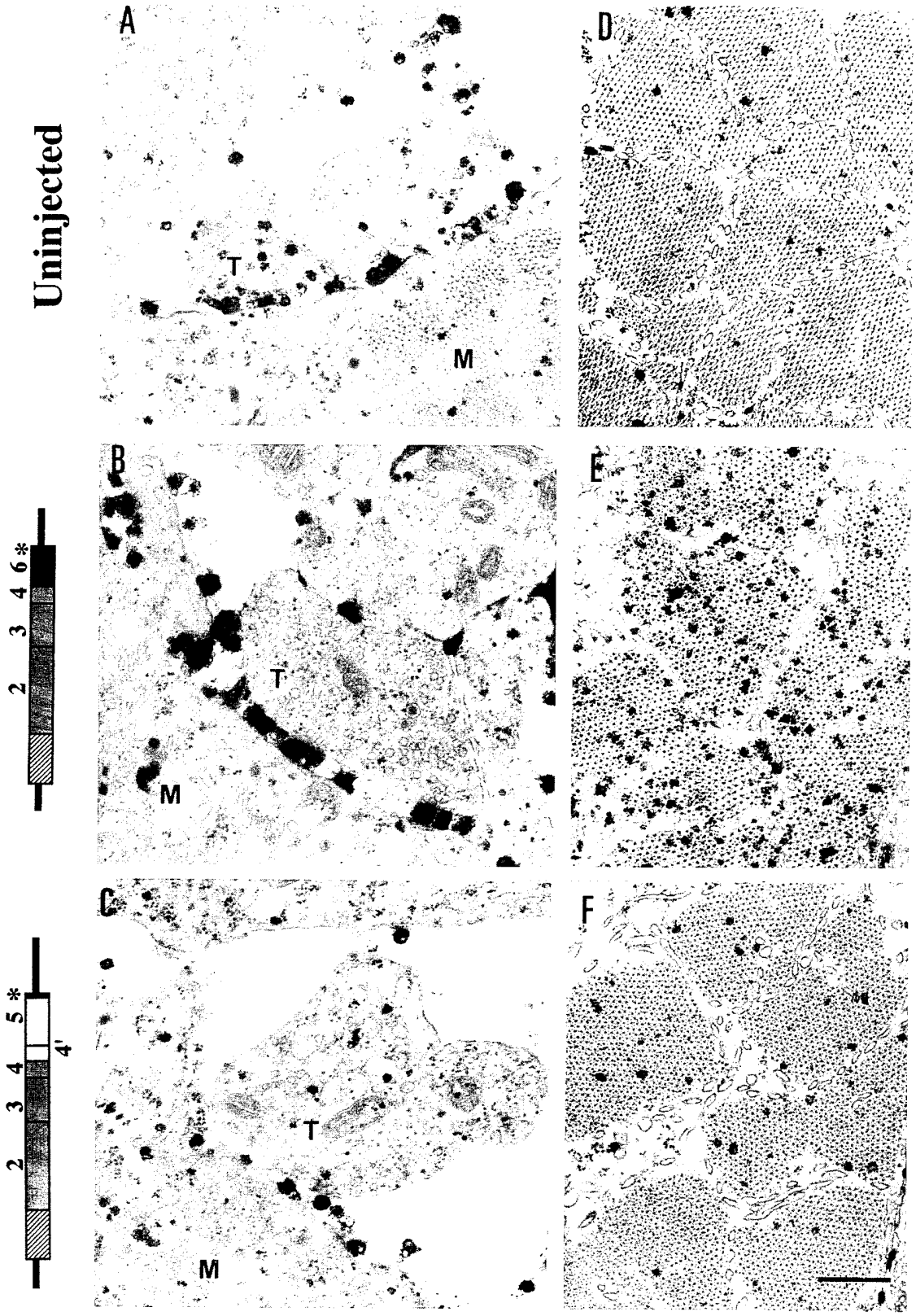

FIG. 3. I4/E5-derived AChE, unlike ACHE-E6, is excluded from NMJs. Two-day-old DNA-injected and control uninjected *Xenopus* embryos raised at 19°C were cytochemically stained for catalytically active AChE and examined by electron microscopy as described in Materials and Methods. Note the significantly enhanced staining, observed as dark electron-dense deposits, and increased size displayed by NMJs from ACHE-E6-injected embryos (B) as compared with those from control (A) and ACHE-I4/E5-injected (C) embryos. Shown are transverse sections of myofibers from control (D), ACHE-E6-injected (E), and ACHE-I4/E5-injected (F) embryos; note the correlation between myotomal and synaptic levels of expression. T, nerve terminal; M, muscle cell. For an explanation of the schematic diagrams, see Fig. 1. Bar = 1 μm.

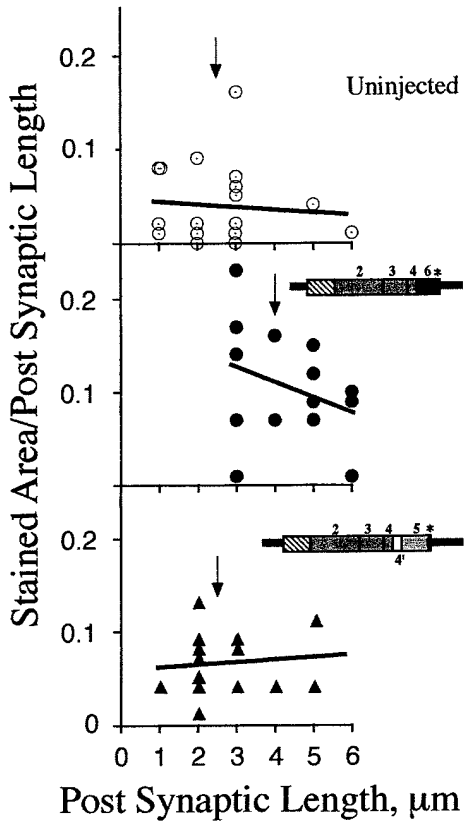

FIG. 4. ACHE-E6 but not ACHE-I4/E5 promotes NMJ maturation. Ratios of AChE-stained area to postsynaptic length as a function of length are presented for cross sections from at least 14 individual NMJs from embryos injected with ACHE-E6 (middle panel) or ACHE-I4/E5 (lower panel) and raised for 2 days at 21°C as compared with controls (upper panel). The solid line represents the best-fit line through the datum points, and the arrows designate the mean postsynaptic length for each microinjection group. Note the rightward shift toward larger NMJs in embryos injected with ACHE-E6 compared with those found in ACHE-I4/E5-injected and control embryos. For an explanation of the schematic diagrams, see Fig. 1.

dence and intensity of staining were higher among the ciliated cells, with some displaying massive apical accumulations of reaction product (Fig. 5). This irregular mosaic of heavily stained cells corresponded well to the punctuated array of stained cells observed in whole-mount embryos (Fig. 2). In uninjected control and ACHE-E6-injected embryos, both types of epidermal cells displayed scant staining, if any (Fig. 5B, D, and F and data not shown).

Ciliated cells were characterized, in addition to their cilia, by their dense cytoplasm, rich accumulation of mitochondria, and the presence of numerous small vesicles, most of which were filled with reaction product in stained cells (Fig. 5). Crystals of reaction product were also observed, however, in the cytoplasm and increased in size and density in a graded fashion toward the apex. In contrast, secretory cells were identified on the basis of their large, distinctive, chondroitin-laden secretory vesicles (Fig. 5) (21), some of which appeared to be fused to the plasma membrane (data not shown). In labelled secretory cells from ACHE-I4/E5-injected embryos, up to 20% of the vesicles stained positively for AChE. However, only an occasional crystal of reaction product could be observed in vesicles from uninjected or ACHE-E6-injected embryos (Fig. 5 and data not shown). No gross morphological features distinguished stained cells or vesicles in ACHE-I4/E5-injected embryos from unstained ones or from those observed in control or ACHE-E6-injected embryos.

The observation that mature secretory vesicles in epidermis of ACHE-I4/E5-injected embryos stained positively for AChE suggested that this enzyme form may be secreted along with the mucus naturally contained within these vesicles. To determine whether AChE was being excreted from the body, healthy neurula-stage embryos either injected with vector or uninjected were incubated overnight and the AChE activity found in the medium was compared with that measured in total homogenates. Only incubation medium from embryos injected with ACHE-I4/E5 DNA displayed significant AChE activity, representing up to 40% of the total measured activity (Fig. 6). Together, these observations indicated that a property or properties intrinsic to the I4/E5-terminal exon confer(s) a signal for epidermal accumulation, polarized subcellular transport, and excretion of AChE. Transfection studies with cultured glioma cells later revealed that secretion of the ACHE-I4/E5 product is a general phenomenon (11a).

Epidermal excretion is associated with readthrough AChE mRNA. Microinjected *Xenopus* embryos have been shown to correctly splice intron I1 from a human AChE promoter-reporter construct to produce catalytically active AChE (1). ACHE-I4/E5 potentially leads to both the mRNA encoding the erythrocyte GPI-linked AChE, by splicing out of intron I4, and/or the complete readthrough mRNA in which the invariant exons continue directly from exon E4 into intron I4 and through it into exon E5 (11) (Fig. 7A). To determine which AChE mRNA(s) was produced in our *Xenopus* embryos, we subjected total RNA extracted from 1-day-old ACHE-E6- or ACHE-I4/E5-injected embryos to RT-PCR with E4-, I4-, E5-, or E6-specific primers (Fig. 7A and see Materials and Methods). When an E6-specific primer pair was employed to analyze RNA from ACHE-E6-injected gastrulae, the expected 482-bp fragment representing full-length ACHE-E6 mRNA was reproducibly observed (Fig. 7B). When RNA from ACHE-I4/E5-injected embryos was subjected to RT-PCR, both the invariant exon E4 and the intronic sequence I4 were detected. However, the E5-specific primer pair repeatedly failed to generate either the 479-bp fragment representing the full-length readthrough mRNA or the 399-bp fragment representing spliced E5-bearing mRNA (Fig. 7B). A parallel reaction using control plasmid DNA and the identical E5-specific primers did yield the 479-bp band, however, validating the efficacy of this primer pair (Fig. 7B). Moreover, this same primer pair has been successfully utilized to detect native E5-carrying mRNAs in human tissues and transfected mammalian cells (11, 11a). These data therefore indicated that the recombinant human AChE activity induced by ACHE-E6 reflected the complete brain-muscle form of AChE, whereas heterologous AChE activity produced in *Xenopus* embryos from ACHE-I4/E5 was derived from a readthrough AChE mRNA which was, at least partially, truncated. This, together with the stop codon included in I4, implied that the polypeptide encoded by ACHE-I4/E5-derived mRNA in *Xenopus* embryos could display biochemical characteristics distinct from both brain-muscle and erythrocyte AChEs.

Unique properties of ACHE-I4/E5-derived AChE. Microinjection of 1 ng of ACHE-E6 DNA induces transiently high levels of catalytically active recombinant human AChE in *Xenopus* embryos (27). When ACHE-I4/E5 was introduced into 1- to 2-cell cleaving embryos, equivalent levels and a similarly transient pattern of heterologous overexpression were observed, peaking at days 1 to 2 postfertilization and receding at day 4 or 5 (Fig. 8A). Overall, development of injected embryos appeared normal through gastrulation, neurulation, hatching,

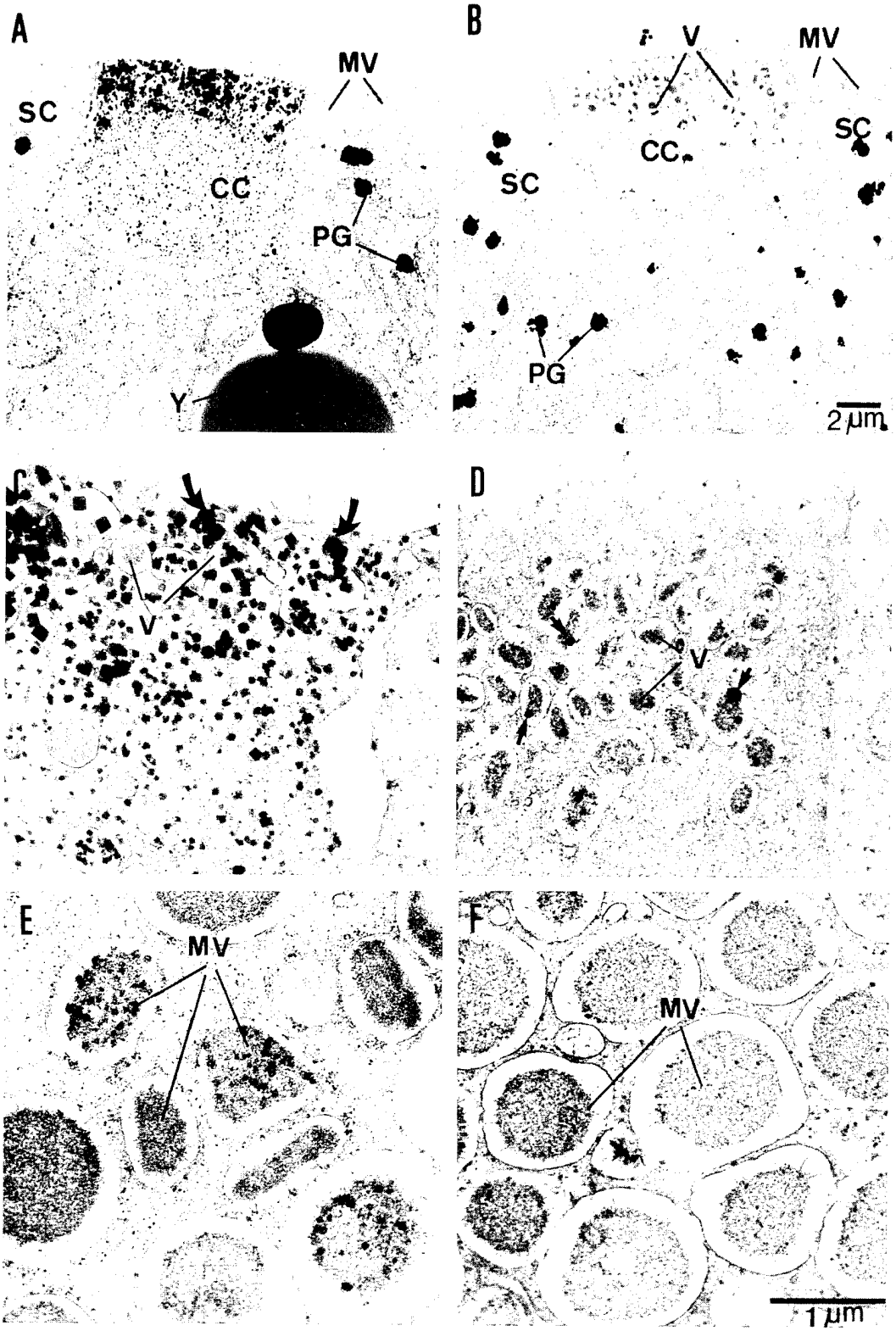

FIG. 5. ACHE-I4/E5-induced AChE accumulates in epidermal cells. Three-day-old DNA-injected and control uninjected *Xenopus* embryos raised at 19°C were fixed and stained for catalytically active AChE as described in Materials and Methods, except that the electron micrographs shown in panels A through D were taken after extending the staining reaction for 2 h. (A and B) Low-magnification image of ciliated (CC) and secretory (SC) cells of *Xenopus* epidermis from ACHE-I4/E5-injected (A) and control (B) embryos. Note the massive accumulation of crystalline electron-dense reaction product at the apex of the CC from an ACHE-I4/E5-injected embryo. Cilia are not visible at this depth. MV, secretory mucous vesicles; PG, pigment granules; V, vesicles; Y, yolk. (C and D) High-magnification image of the apical ridge of a ciliated epidermal cell from ACHE-I4/E5-injected (C) and control, uninjected (D) embryos. Note the appearance of crystals both within V (arrows) and within the cytoplasm of the injected embryo. (E and F) High-magnification image of MV from ACHE-I4/E5-injected (E) and control, uninjected (F) embryos. Note that only MV from the ACHE-I4/E5-injected embryo demonstrate accumulation of reaction product in exocytotic vesicles adjacent to the external cell membrane.

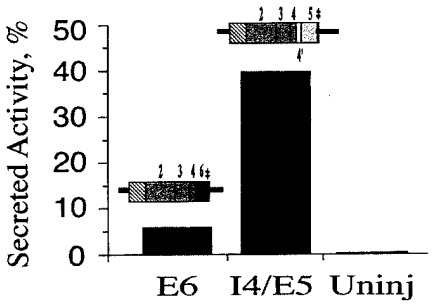

FIG. 6. ACHE-I4/E5 but not ACHE-E6 is excreted into the external medium. Fractions of total recovered AChE activity (medium plus whole-cell extract) in the external growth medium are presented for 20 DNA-injected (E6 and I4/E5) or control (Uninj) embryos following incubation of neurula-stage embryos overnight (day 1→day 2) in fresh buffer. Note that only embryos expressing ACHE-I4/E5 DNA displayed significant secretion of AChE into the surrounding medium. For an explanation of the schematic diagrams, see Fig. 1.

and the acquisition of motor function. Although mock injections indicated that microinjection may slightly retard growth and the accumulation of endogenous AChE (data not shown), quantitative RT-PCR performed with primers specific for XmyoD (8) and *Xenopus* GATA-2 (34) indicated that no global changes occurred in the levels of endogenous RNAs encoding these myogenesis- and hematopoiesis-promoting proteins in AChE DNA-injected embryos (data not shown).

To compare the hydrodynamic properties of the recombinant human enzymes, we performed sequential extractions of gastrula-stage embryos into low-salt, low-salt–detergent, and high-salt buffers. AChE activity from embryos injected with ACHE-E6 DNA consistently partitioned into both the low-salt (55%) and low-salt–detergent (35%) fractions. In contrast, activity from embryos injected with ACHE-I4/E5 was 85 to 90% solubilized in the low-salt step (Fig. 8B). In sucrose density gradient centrifugation, a single peak between 3 and 4 S was observed for the ACHE-E6- and ACHE-I4/E5-derived enzymes, both of which cosedimented with the clearly monomeric recombinant enzyme produced in bacteria (reference 27 and data not shown). This is consistent with a monomeric configuration for the cell-associated recombinant molecules. However, by nondenaturing polyacrylamide gel electrophoresis, catalytically active AChE from ACHE-I4/E5-injected embryos migrated significantly faster than the bands representing either recombinant human AChE from ACHE-E6-injected embryos or native AChE from human brain or erythrocytes (Fig. 8C). When electrophoresis was performed in the absence of detergent, no significant shift in the migration of ACHE-I4/E5-derived bands was observed, suggesting that this molecule represented a nonhydrophobic AChE species (Fig. 8C).

DISCUSSION

In transiently transgenic *X. laevis* embryos, AChE mRNA bearing the alternative 3′ exon E6 induced specific accumulation of human AChE in muscle and NMJs and enhanced NMJ development. Replacement of E6 with an in-frame pseudointronic sequence of similar size directed production of equal amounts of a soluble enzyme species that was amassed in epidermal cells and excreted into the external culture medium and which was not incorporated into muscle or NMJs and did not affect their development. These observations suggest that the 3′-terminal exons encoding the various AChE subtypes played indispensable roles in their NMJ or epidermal localization.

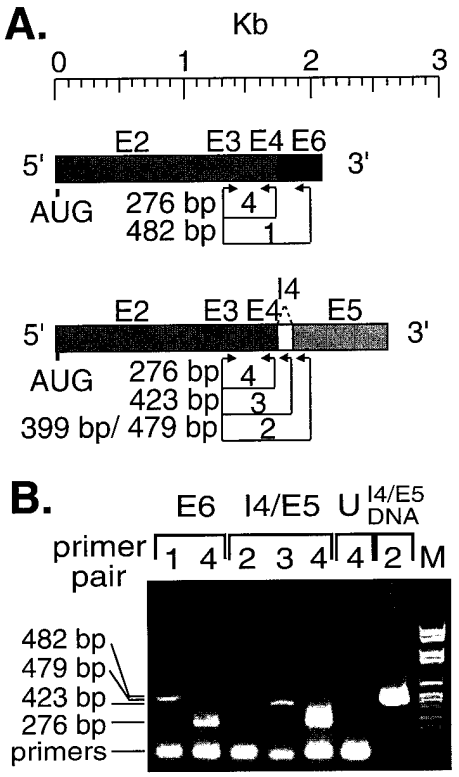

FIG. 7. Epidermal expression of ACHE-I4/E5 is associated with presence of a 3′-truncated readthrough mRNA. (A) Schematic representation of possible AChE mRNA products. Schemes present ACHE-E6 (upper) and ACHE-I4/E5 (lower) mRNA; I4 (indicated by dashed triangle) may be spliced out of the primary ACHE-I4/E5 transcript or retained in the mature mRNA. The expected product lengths of PCR primer pairs 1 through 4 (see Materials and Methods) are indicated to the left of the arrows. (B) RT-PCR. RT-PCR was performed on total RNA from 1-day-old *Xenopus* embryos injected with the ACHE-E6 (E6) or ACHE-I4/E5 (I4/E5) DNA constructs. Uninjected embryos (U) served as the control. PCR products were electrophoresed as previously described (1), and their lengths were evaluated by markers (M) of known size. PCR analysis of DNA performed with the ACHE-I4/E5 DNA construct (I4/E5 DNA) confirmed the ability of primer pair no. 2 to yield a PCR product. Control reactions, without RT, did not yield amplification products, proving the absence of contaminating DNA sequences (data not shown).

Differential posttranscriptional management of alternative AChE forms. The native human AChE promoter includes consensus recognition sites for transcription factors indicative of tissue-specific regulation of transcription (1). However, the CMV promoter used to direct the expression of AChE in *X. laevis* is pan-active (26), and our in situ hybridization suggests that it is expressed in the embryos in a nonspecific manner relatively early in development. This would explain the high levels of heterologous enzyme observed at the gastrula stage (day 1). Thus, the tissue-specific accumulation of alternative heterologous AChEs reflects differential posttranscriptional or posttranslational management of their respective RNA and/or protein products. Stabilization of AChE mRNA was recently shown to account for the increased AChE activity accompanying differentiation of cultured myoblasts (7). Our present observations add differential stability of alternative AChE polypeptides as a potential factor in the tissue-specific accumulation of these AChE forms.

In vivo, AChE is subject to tissue-specific and developmentally regulated posttranscriptional and posttranslational processing, which gives rise to a complex array of molecular forms varying in their extents of oligomeric assembly, association

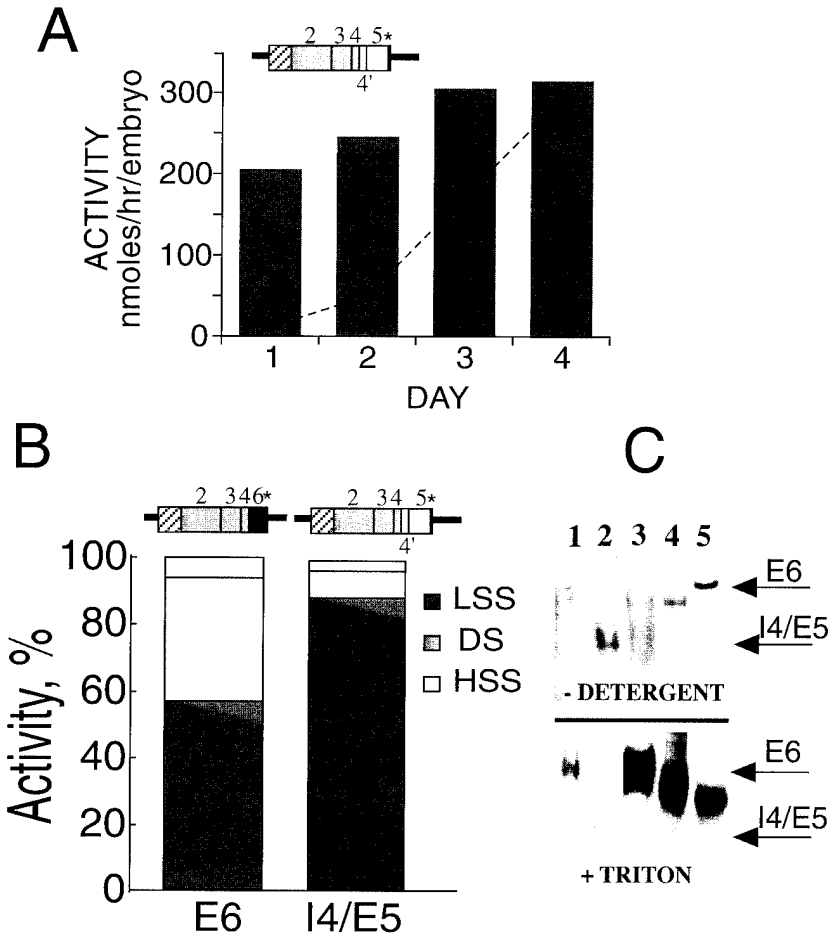

FIG. 8. ACHE-I4/E5 expression yields a soluble, hydrophilic enzyme with fast electrophoretic mobility. (A) Transient expression. Cleaving embryos (1 to 2 cells) injected with approximately 1 ng of ACHE-I4/E5 DNA under control of the CMV promoter were grown for 1 to 4 days at 20°C. Homogenates were assayed for acetylthiocholine hydrolyzing activity. The columns represent total AChE activity measured in DNA-injected embryos. The squares (□) represent endogenous AChE activity measured in age-matched control uninjected embryos. Note the developmentally regulated increase in endogenous AChE levels and the concomitant decline in the relative extent of overexpression from days 1 to 4. The data represent averages of three separate groups of four embryos from a single microinjection experiment. (B) Differential solubility. Sequential extractions of 10 1-day-old DNA-injected embryos into low-salt-soluble (LSS), low-salt–detergent-soluble (DS), and high-salt-soluble (HSS) fractions were performed as described in Materials and Methods. The columns represent the relative fractions of the total summed activities extracted at each step for a single representative experiment. Endogenous *Xenopus* AChE activities were considered negligible and were ignored. Note the predominantly low-salt-soluble nature of the ACHE-I4/E5 (I4/E5) product(s) as compared with that of the recombinant human AChE as encoded by ACHE-E6 (E6). (C) Distinct electrophoretic mobility of alternative AChEs. Nondenaturing gel electrophoresis was performed as described in Materials and Methods in the presence or absence of 0.5% Triton X-100. Lanes 1 and 2, ACHE-E6- and ACHE-I4/E5-derived products, respectively, as expressed in *Xenopus* embryos (1 day postfertilization); lane 3, purified human erythrocyte AChE; lanes 4 and 5, recombinant human AChE-E6 (E6) as expressed in human 293 cells and *E. coli*, respectively. Note that the ACHE-I4/E5 (I4/E5) product(s) migrates significantly faster than all the other AChE forms both in the presence and in the absence of detergent. No band representing endogenous *Xenopus* AChE could be detected at this time point. The smearing of activity in lane 3 of the no-detergent (−) gels probably represents aggregation of the hydrophobic GPI-linked AChE erythrocyte dimer in the absence of Triton X-100. For an explanation of the schematic diagrams in panels A and B, see Fig. 1.

with noncatalytic subunits, hydrodynamic properties, and sites of subcellular localization (17). One level at which this diversity is controlled appears to be alternative splicing of 3′ exons (11, 15, 28). Transfections of AChE-coding sequences into mammalian cells indicated that alternative splicing alone could account for these multiple molecular forms of AChE (5, 12, 14, 16). Our results extend these conclusions by demonstrating that alternative splicing may dictate the final complement of specific AChE catalytic subunits available to particular cell types through selective management of specific AChE mRNAs and AChE polypeptides. Translated or nontranslated AChE mRNA, stable or unstable AChE mRNA, or polypeptides may dictate the final complement of specific AChE catalytic subunits expressed in particular cell types.

3′ exon E6 defines an indispensable motif for synaptic accumulation of AChE. Compartmentalized biosynthesis of the nicotinic acetylcholine receptor (reference 18 and reviewed in reference 4) as well as other NMJ proteins (22, 24) suggests that an intricate network of factors are at work to produce and localize NMJ proteins around junctional regions prior to their active accumulation at the NMJ. This network could potentially include *cis*-acting elements intrinsic to the mRNA or primary amino acid sequences of NMJ-targeted proteins as well as *trans*-acting cellular components capable of anchoring or translocating these molecules within the muscle. An example of a *trans*-acting factor controlling the translocation and subcellular anchoring of specific mRNAs is the 69-kDa protein associated with the developmentally regulated accumulation of two vegetal mRNAs in *Xenopus* oocytes (23). Our present findings demonstrate that the transiently transgenic embryos of *X. laevis* can be further employed to search for *cis*- and *trans*-acting factors controlling the management of proteins from

heterologous species. Recent work demonstrating the spatially restricted biosynthesis of AChE in avian muscle predicts the existence of localized cellular factors recognizing AChE and/or its encoding mRNA (9). In the present study, we observed that exon E6 and/or its encoded peptide participate in forming a recognition signal(s) through which such putative cellular elements might mediate accumulation and subcellular localization of AChE. Search of the Genetics Computer Group data bank did not reveal significant homologies between the E6-encoded peptide and any protein except the related acetylcholine-hydrolyzing enzyme butyrylcholinesterase. However, it is interesting that this enzyme, which has high homology (56%) to AChE in the corresponding C-terminal domain, also accumulated in NMJs of microinjected *Xenopus* embryos (reference 29 and unpublished observations). Moreover, both these enzymes are also found in brain nerve-nerve synapses (29). Thus, our findings suggest that exon E6 defines a conserved motif for muscle accumulation and trafficking of cholinesterases into synapses but not necessarily a general signal for targeting proteins to NMJs. Further experiments will be required to determine whether E6 is sufficient to direct synaptic accumulation of unrelated proteins to the NMJ as well.

A novel I4/E5-derived readthrough form of human AChE. An mRNA representing the I4 readthrough transcript in mouse tissues was reported but is considered absent in human cells. This difference was attributed to an inherent property of the human AChE nucleotide sequence (16). Nonetheless, we have recently observed, using RT-PCR and I4-specific primers, mRNA carrying the retained I4 sequence, in addition to E5 and E6, in several tumor cell lines of human origin (11) as well as in numerous mouse tissues (data not shown). That study did not, however, reveal whether this mRNA species is translated into protein and what the properties of the putative human readthrough AChE might be. In the present work, RT-PCR indicated the presence of a potentially truncated readthrough mRNA derived from ACHE-I4/E5 in microinjected *Xenopus* embryos. The protein translated from this readthrough RNA would lack the cleavable hydrophobic E5 stretch, precluding GPI linkage and leaving the relatively hydrophilic I4-encoded peptide. This could explain the high solubility of catalytically active AChE derived from ACHE-I4/E5 DNA.

When DNA encoding the mouse readthrough mRNA, where a nonsense codon appears 40 nucleotides downstream of the E4/I4 boundary, was expressed in COS cells, 97% of the total activity was soluble and secreted into the culture medium (16). The present study suggests that a truncated human readthrough AChE mRNA might also be translatable, giving rise to a catalytically active, nonmuscle, secretory form of AChE in *X. laevis*. However, the overall timing and levels of ACHE-I4/E5 expression in the embryos as assessed in total homogenates indicate generally similar stability of its mRNA and protein product as compared with those derived from ACHE-E6. Therefore, these observations may offer the first indications for the existence of yet another stable human AChE subtype representing the common exons E2, E3, and E4 and the pseudointron I4.

The human readthrough AChE mRNA (11) carries a 9-nucleotide sequence (5′-ACCTGCCCA-3′) beginning 18 nucleotides downstream of the I4/E5 boundary that is identical (in 8 of 9 bp) to that contained within a 19-bp consensus mRNA-destabilizing site (3). This sequence mediates endonucleolytic cleavage of RNA in *X. laevis* and *Drosophila melanogaster* and could explain the appearance of a truncated RNA. Indeed, when DNA encoding GPI-linked *Drosophila* AChE was expressed in microinjected *Xenopus* oocytes, a hydrophobic, but nonglypiated, membrane-associated enzyme was obtained. However, deletion of the sequence encoding the 27-amino-acid hydrophobic C-terminal peptide yielded a soluble, secreted AChE (6). Experiments to determine the precise 3′ and/or C-terminal limit of ACHE-I4/I5-derived mRNA and its polypeptide product are currently in progress.

Secretory AChE-I4/E5 may reflect an in vivo entity. It is yet uncertain whether generation of the unusual ACHE-I4/E5-derived protein and its accumulation in epidermis reflect natural events in the biosynthesis of human AChE or an anomalous expression pattern for this AChE in *X. laevis*. However, the putative AChE generated from ACHE-I4/E5 in *X. laevis* is catalytically indistinguishable from the ACHE-E6-derived enzyme (26a) and should be very close in size to the 583-amino-acid brain-muscle form. Therefore, the presence of such a discrete molecule in native human tissues would be difficult to detect and might easily go unnoticed. Moreover, a soluble AChE form with a distinct migration profile in isoelectric focussing was observed in the cerebrospinal fluid of patients with Alzheimer's disease (19), and soluble AChE monomers were reported in the serum of patients with carcinomas (33). Thus, the distinct migration of the short readthrough AChE derived from ACHE-I4/E5 in nondenaturing gel electrophoresis may provide a tool to search for a naturally occurring form of this enzyme species in humans.

In conclusion, we have used microinjected *Xenopus* embryos for transgenic overexpression of DNA constructs encoding variable C-terminal alternative forms of AChE. We established an NMJ-accumulating role for the 3′ exon E6 and observed epidermal expression and active secretion for human AChE encoded by a construct carrying the alternative 3′ region, I4/E5. ACHE-I4/E5 displayed hydrophilic properties and fast electrophoretic migration, reminiscent of a human AChE form reported in Alzheimer's disease. It was further excluded from muscle and NMJ structures and, in contrast with ACHE-E6, did not enhance NMJ development. These findings demonstrate that alternative 3′ exons in the AChE gene specify distinct tissue-recognizable signals and suggest the use of transiently transgenic *Xenopus* embryos for disclosing cell type specificities and biological roles of gene products from heterologous species.

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REFERENCES

1. **Ben Aziz-Aloya, R., S. Seidman, R. Timberg, M. Sternfeld, H. Zakut, and H. Soreq.** 1993. Expression of a human acetylcholinesterase promoter-reporter construct in developing neuromuscular junctions of *Xenopus* embryos. Proc. Natl. Acad. Sci. USA **90**:2471–2475.
2. **Billett, F. S., and R. P. Gould.** 1971. Fine ultrastructural changes in the differentiating epidermis of *Xenopus laevis* embryos. J. Anat. **108**:465–480.
3. **Brown, B. D., H. D. Zipkin, and R. Harland.** 1993. Sequence-specific endonucleolytic cleavage and protection of mRNA in *Xenopus* and *Drosophila*. Genes Dev. **7**:1620–1631.
4. **Changeux, J. P.** 1991. Compartmentalized transcription of acetylcholine receptor genes during motor endplate epigenesis. New Biol. **3**:413–429.
5. **Duval, N., J. Massoulie, and S. Bon.** 1992. H and T subunits of acetylcholinesterase from *Torpedo*, expressed in COS cells, generate all types of molecular forms. J. Cell. Biol. **118**:641–653.
6. **Fournier, D., A. Mutero, and D. Rungger.** 1992. *Drosophila* acetylcholinesterase: expression of a functional precursor in *Xenopus* oocytes. Eur. J. Biochem. **203**:513–519.

7. **Fuentes, M. E., and P. Taylor.** 1993. Control of acetylcholinesterase gene expression during myogenesis. Neuron **10:**379–387.
8. **Hopwood, N. D., A. Pluck, and J. B. Gurdon.** 1989. MyoD expression in the forming somites is an early response to mesoderm induction in *Xenopus* embryos. EMBO J. **8:**3409–3417.
9. **Jasmin, B. J., R. K. Lee, and R. L. Rotundo.** 1993. Compartmentalization of acetylcholinesterase mRNA and enzyme at the vertebrate neuromuscular junction. Neuron **11:**467–477.
10. **Karnovsky, M. J., and L. Roots.** 1964. A direct coloring method for cholinesterases. J. Histochem. Cytochem. **12:**219–221.
11. **Karpel, R., R. Ben Aziz-Aloya, M. Sternfeld, G. Ehrlich, D. Ginzberg, P. Tarroni, F. Clementi, H. Zakut, and H. Soreq.** 1994. Expression of three alternative acetylcholinesterase messenger RNAs in human tumor cell lines of different tissue origins. Exp. Cell Res. **210:**268–277.

11a. **Karpel, R., et al.** Submitted for publication.

12. **Krejci, E., F. Coussen, N. Duval, J.-M. Chatel, C. Legay, M. Puype, J. Vandekerckhove, J. Cartaud, S. Bon, and J. Massoulie.** 1991. Primary structure of a collagenic tail peptide of *Torpedo* acetylcholinesterase: co-expression with catalytic subunit induces the production of collagen-tailed forms in transfected cells. EMBO J. **10:**1285–1293.
13. **Legay, C., S. Bon, and J. Massoulie.** 1993. Expression of a cDNA encoding the glycolipid-anchored form of rat acetylcholinesterase. FEBS Lett. **315:** 163–166.
14. **Legay, C., S. Bon, P. Vernier, F. Coussen, and J. Massoulie.** 1993. Cloning and expression of a rat acetylcholinesterase subunit: generation of multiple molecular forms, complementarity with a Torpedo collagenic subunit. J. Neurochem. **60:**337–346.
15. **Li, Y., S. Camp, T. L. Rachinsky, D. Getman, and P. Taylor.** 1991. Gene structure of mammalian acetylcholinesterase. Alternative exons dictate tissue-specific expression. J. Biol. Chem. **266:**23083–23090.
16. **Li, Y., S. Camp, and P. Taylor.** 1993. Tissue-specific expression and alternative messenger RNA processing of the mammalian acetylcholinesterase gene. J. Biol. Chem. **268:**5790–5797.
17. **Massoulie, J., L. Pezzementi, S. Bon, E. Krejci, and F. M. Vallette.** 1993. Molecular and cellular biology of cholinesterases. Prog. Neurobiol. **41:**31–91.
18. **Merlie, J., and J. R. Sanes.** 1985. Concentration of acetylcholine receptor mRNA in synaptic regions of adult muscle fibers. Nature (London) **317:**66–68.
19. **Navaratnam, D. S., J. D. Priddle, B. McDonald, M. M. Esiri, J. R. Robinson, and A. D. Smith.** 1991. Anomalous molecular form of acetylcholinesterase in cerebrospinal fluid in histologically diagnosed Alzheimer's disease. Lancet **337:**447–450.
20. **Neville, L. F., A. Gnatt, Y. Loewenstein, S. Seideman, G. Ehrlich, and H. Soreq.** 1992. Intramolecular relationships in cholinesterases revealed by oocyte expression of site-directed and natural variants of human BCHE. EMBO J. **11:**1641–1649.
21. **Nishikawa, S., and F. Sasaki.** 1993. Secretion of chondroitin sulfate from embryonic epidermal cells in *Xenopus laevis*. J. Histochem. Cytochem. **9:**1373–1381.
22. **Pavlath, G. K., K. Rich, S. G. Webster, and H. M. Blau.** 1989. Localization of muscle gene products in nuclear domains. Nature (London) **337:**570–573.
23. **Pressman-Schwartz, S., L. Aisenthal, Z. Elisha, F. Oberman, and J. K. Yisraeli.** 1992. A 69-kDa RNA-binding protein from *Xenopus* oocytes recognizes a common motif in two vegetally localized maternal mRNAs. Proc. Natl. Acad. Sci. USA **89:**11895–11899.
24. **Ralston, E., and Z. W. Hall.** 1989. Transfer of a protein encoded by a single nucleus to nearby nuclei in multinucleated myotubes. Nature (London) **244:**1066–1069.
25. **Salpeter, M.** 1967. Electron microscope radioautography as a quantitative tool in enzyme cytochemistry. I. The distribution of acetylcholinesterase at motor endplates of a vertebrate twitch muscle. J. Cell Biol. **32:**379–389.
26. **Schmidt, E. V., G. Christoph, R. Zeller, and P. Leder.** 1990. The cytomegalovirus enhancer: a pan-active control element in transgenic mice. Mol. Cell. Biol. **10:**4406–4411.

26a. **Schwarz, M., Y. Loewenstein-Lichtenstein, D. Glick, J. Liao, B. Norgaard-Pedersen, and H. Soreq.** Catalysis by human cholinesterase variants dissected by successive organophosphorus inhibition and oxime reactivation. Mol. Brain Res., in press.

27. **Seidman, S., R. Ben Aziz-Aloya, R. Timberg, Y. Loewenstein, B. Velan, A. Shafferman, J. Liao, B. Norgaard-Pedersen, U. Brodbeck, and H. Soreq.** 1994. Overexpressed monomeric human acetylcholinesterase induces subtle ultrastructural modifications in developing neuromuscular junctions of *Xenopus laevis* embryos. J. Neurochem. **62:**1670–1681.
28. **Sikorav, J. L., N. Duval, A. Anselmet, S. Bon, E. Krejci, C. Legay, M. Osterlund, B. Reimund, and J. Massoulie.** 1988. Complex alternative splicing of acetylcholinesterase transcripts in *Torpedo* electric organ: primary structure of the precursor of the glycolipid-anchored dimeric form. EMBO J. **7:**2983–2993.
29. **Soreq, H., R. Beeri, S. Seidman, R. Timberg, Y. Loewenstein, M. Sternfeld, and C. Andres.** 1994. Modulating cholinergic neurotransmission through transgenic overexpression of human cholinesterases, p. 84–87. *In* R. E. Becker and E. Giacobini (ed.), Pharmacological basis of cholinergic therapy in Alzheimer's disease. Birkhauser, Boston.
30. **Soreq, H., R. Ben-Aziz, C. Prody, S. Seidman, A. Gnatt, L. Neville, J. Lieman-Hurwitz, E. Lev-Lehman, D. Ginzberg, Y. Lapidot-Lifson, and H. Zakut.** 1990. Molecular cloning and construction of the coding region for human acetylcholinesterase reveals a G,C rich attenuating structure. Proc. Natl. Acad. Sci. USA **87:**9688–9692.
31. **Soreq, H., and H. Zakut.** 1993. Human cholinesterases and anticholinesterases. Academic Press, San Diego, Calif.
32. **Velan, B., C. Kronman, H. Grosfeld, M. Leitner, Y. Gozes, Y. Flashner, T. Sery, S. Cohen, R. Ben-Aziz, S. Seidman, A. Shafferman, and H. Soreq.** 1991. Recombinant human acetylcholinesterase is secreted from transiently transfected 293 cells as a soluble globular enzyme. Cell. Mol. Neurobiol. **11:**143–156.
33. **Zakut, H., L. Even, S. Birkenfeld, G. Malinger, R. Zisling, and H. Soreq.** 1988. Modified properties of serum cholinesterases in primary carcinomas. Cancer **61:**727–737.
34. **Zon, L. I., C. Mather, S. Burgess, M. E. Bolce, R. M. Harland, and S. H. Orkin.** 1991. Expression of GATA-binding proteins during embryonic development in *Xenopus laevis*. Proc. Natl. Acad. Sci. USA **88:**10642–10646.

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Mutations and impaired expression in the ACHE and BCHE genes: neurological implications

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Summary – The acetylcholine hydrolysing cholinesterases control the termination of cholinergic signalling in multiple tissues and are targets for a variety of drugs, natural and man-made poisons and common insecticides. Molecular cloning and gene mapping studies revealed the primary structure of human acetyl- and butyrylcholinesterase and localized the corresponding ACHE and BCHE genes to the chromosomal positions 3q26-ter and 7q22, respectively. Several different point mutations in the coding region of BCHE were found to be particularly abundant in the Israeli population. Analytical expression studies in microinjected *Xenopus* oocytes have demonstrated that the biochemical properties of cholinesterases may be modified by rationalized site-directed mutagenesis and in chimeric ACHE/BCHE constructs. These properties are differently altered in the various allelic BCHE variants, conferring resistance to several anti-cholinesterases, which may explain the evolutionary emergence of these multiple alleles. At the clinical level, abnormal expression of both ACHE and BCHE and the *in vivo* amplification of the ACHE and BCHE genes has been variously associated with abnormal megakaryocytopoiesis, leukemias and brain and ovarian tumors. Moreover, antisense oligonucleotides blocking the expression of these genes were shown to interfere with hemocytopoiesis in culture, implicating these genes in cholinergic influence on cell growth and proliferation.

ACHE / BCHE / mutations / neurological diseases

Introduction

Two single-copy genes, designated ACHE and BCHE, encode the acetylcholine-hydrolysing enzymes acetyl- and butyrylcholinesterase (AChE and BuChE, respectively). The clinical role of AChE needs no introduction to neurologists; however, the role of BuChE is less obvious. In addition to hydrolyzing acetylcholine, though at a considerably slower pace than AChE [47], BuChE acts as a scavenger of poisons targeted at acetylcholine binding proteins. These include, in particular, AChE and the various acetylcholine receptors [47]. The human ACHE and BCHE genes were both molecularly cloned [42, 48] and mapped to the chromosomal positions 7q22 [20, 22] and 3q26 [5, 23], respectively. Because of the physiological role of the enzymes, mutations in both of the cholinesterase genes (CHEs) are physiologically important, as indicated by the fact that reduced cholinesterase (ChE) enzyme activity due to natural or man-produced inhibitors has severe neurological consequences. In the following, we discuss the current state of information regarding the basis for these consequences.

BuChE mutations: causes for congenital susceptibility to altered drug responses

Twenty-two different mutations in the coding region of the human BCHE gene have been identified in the human population since the gene was cloned [41]. Some of these alter various amino acids and introduce different phenotypic changes in the resultant enzyme [37]. Other mutations introduce stop signals into the mRNA transcript, producing incomplete, "silent" (inactive), BuChE [40]. The active variants are far more abundant in the population than the silent ones, all of which together are only 0.0001% of homozygotes [55], which suggests that the frequent phenotypically-effective variants confer a selection advantage for

development. The presumption is, that random mutation would produce more silent mutations, and, as these are rare, they must be selected against.

Cholinesterase genes were analyzed by molecular cloning, library screening and polymerase chain reactions (PCR) amplification, all as detailed elsewhere [20, 23, 42, 48]. Positions of substituted amino acid residues within the mature AChE protein were determined by superimposing the primary amino acid sequence of human AChE [48] onto the crystal structure of *Torpedo* AChE [52], using the Insight program (Biosym) on a Silicon Graphics computer as detailed in [49]. For BuChE mutations, we employed the recent computer model of human BuChE [25].

The currently available data on mutations in the coding region of the human BCHE gene and their phenotypic consequences are presented in table I and figure 1 and the different mutations causing "silent" BuChE phenotypes are superimposed in figure 2 on the ribbon model of the BuChE protein [25]. It is interesting to note that several of these BuChE variants (*eg* Asp70 → Gly) were simultaneously discovered on two continents, while many others have been detected only once. The search for new variants was not random; mutations in BCHEcDNA or BCHE genes were sought in DNA samples taken from individuals who expressed the variant phenotypes [38, 40, 43] or from tumors presenting biochemical alterations in the BuChE protein [23, 37].

Several of the variant BuChE proteins display an inability to hydrolyze the muscle relaxant succinylcholine [37, 55]. Consequently, patients with such variant BuChEs may suffer prolonged apnea when given standard doses of succinylcholine during anesthesia [26]. The low serum levels of BuChE activity characteristic of several of these variants also implies that tissue types to which ChE activity is essential will be subject to severe stress under exposure of individuals carrying these variants to organophosphorous (OP) insecticides designed to block ChE activities, or by naturally occurring ChE inhibitors.

A single point mutation in the ACHE gene: immunogenic implications

In contrast to the multitude of point mutations in the BCHE gene, records of phenotypically effective variants in the ACHE gene are so far limited to a single point mutation. This was discovered in several steps. The human ACHE gene was first demonstrated to present two co-dominant alleles in a single genetic locus [13]. Recently, a common polymorphism was reported in the ACHE gene, in which His322 is replaced by asparagine due to a C → A substitution [31]. Furthermore, this allele has been identified as the basis of the Yt^b blood group [50], for which an incidence of 5% has been determined in the general Caucasian population [29] and a considerably higher incidence in Israel [28].

The genomic position of the Yt mutation is shown in figure 1. In view of the complete agreement between the original observation of phenotypic diversity and the recent findings on the Yt mutation, it appears that the ACHE gene is much less susceptible to mutation than the related BCHE gene.

Discussion

The plethora and frequency of BuChE variants as compared with the genetic stability of AChE undoubtedly reflects the different levels of importance of these two enzymes. Thus, the essential function of AChE in the termination of neurotransmission precludes mutations that compromise the activity of AChE, as these are undoubtedly lethal. In contrast, the scavenging role, which has been proposed for BuChE, may even lead to a positive selection pressure for proteins with modified properties when these confer resistance to specific cholinergic poisons. However, we posit two caveats: the extreme rarity of the silent BuChE mutations strongly suggests a role for BuChE that perhaps has not yet been identified, and selection does not necessarily render BuChE variants advantageous when faced with man-made ChE inhibitors.

The body's first line of defence against anti-ChEs is plasma BuChE, which scavenges the agent. Congenital variations of BuChE, however, may impair this protective property. Excess ChE inhibitors that have not been eliminated by the BuChE screen, upon reaching neuromuscular junctions (NMJs) may block diaphragm muscles, with a lethal effect [14]. Neurological implications of BuChE mutations, include congenital susceptibility to cholinergic drugs and poisons. Such poisons include primarily OP insecticides, the inhibition by which causes a very specific re-

Table I. Human butyrycholinesterase variants and their phenotypes.

No[a]	*Mutated nucleotide*	*Altered amino acids*	*Phenotype*	*Reference*
1	Wildtype	None	Normal	Prody *et al* (1987) Lockridge *et al* (1987)
2	ATT TT	I6-ter	Silent (frameshift)	Primo-Parmo *et al* (1992)
3	CCT TCT	P37S	Silent	Primo-Parmo *et al* (1992)
4	GAT GGT	D70G	Resistance to succinyl choline and other ligands	Gnatt *et al* (1990) Neville *et al* (1990b)
5	TAT CAT	Y114H	No significant effect, when alone	Neville *et al* (1992)
6	GGT GAT	G115D	Silent	Primo-Parmo *et al* (1992)
7	GGT GGAG	G117-ter	Silent (frameshift)	Noguiera *et al* (1990)
8	GTG ATG	V142M	H-variant (10% specific activity)	Lockridge (1990)
9	GAT GGT	D170G	Silent	Primo-Parmo *et al* (1992)
10	AGT GGT	S198G	Silent	Primo-Parmo *et al* (1992)
11	ACG ATG	T243M	Fluoride-1 (Km variant)	Nogueira *et al* (1992)
12	Alu insert (nt 1062-1077)	D301-ter	Silent (frameshift)	Soreq and Zakut (1993)
13	ACC AACC	T315-ter	Silent (frameshift)	Primo-Parmo *et al* (1992)
14	GGA CGA	G356R	Silent	Primo-Parmo *et al* (1992)
15	GGT GTT	G390V	Fluoride-2 (Km variant)	Bartels *et al* (1992a)
16	TCG CCC	S425P	When coupled with G70, full resistance to succinyl choline and dibucaine	Gnatt *et al* (1990)
17	TGG CGG	W471R	Silent	Primo-Parmo *et al* (1992)
18	GAA GTA	E497V	J variant (33% specific activity)	Bartels *et al* (1992a)
19	TAT TAA	Y500-ter	Silent	Primo-Parmo *et al* (1992)
20	CAA CTA	Q508L	Silent	Primo-Parmo *et al* (1992)
21	GCA ACA	A539T	K variant (70% specific activity)	Bartels *et al* (1992b)
22	TTT TAT	F561Y	No significant effect when alone	Gnatt *et al* (1990)

[a]Mutant number refers to figure 1.

action of the OP compound with the active site serine of ChEs [3]. Blocking of ChEs leads to acetylcholine (ACh) accumulation [2], synaptic excitation and subsequent paralysis. Delayed neurotoxicity includes damage to the peripheral nervous system [1], although the mechanism of this effect is unknown. Clinical symptoms of acute OP poisoning include, in the following

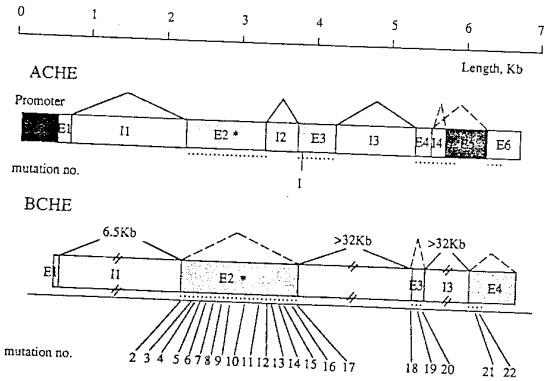

Fig 1. The organization of the ACHE (upper) and BCHE (lower) genes. Note the arrangement of exons (E) and introns (I) and the positions of the natural mutations, numbered as in table I. Above the bars representing the genes are constant (solid lines) and alternative (broken lines) sequences that are excised from the RNA transcripts prior to translation into a tissue- or subcellular component-specific enzyme form.

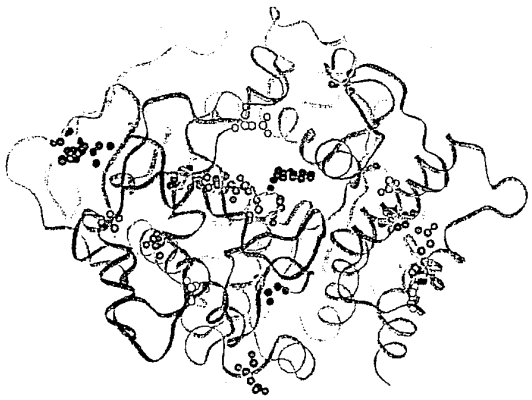

Fig 2. Locations of butyrylcholinesterase mutations. The 22 mutated amino acid residues or the residue corresponding to a mutated codon are shown as space-filling atoms superimposed on the backbone of the BuChE molecule. Note the distribution of the mutations over the entire protein molecule.

order: flushing, dry mouth, fasciculations, tremor, restlessness, agitation, ataxia [34] weakness, convulsions, coma and death [19]. Immediate treatment aimed at restoration of cholinergic function terminates all of these symptoms, although, paradoxically, the levels of plasma BuChE and red blood cell AChE remain low for up to 3 and 12 weeks, respectively [35]. This suggests a much faster regeneration of AChE at the NMJ, but this has not been documented.

Treatment of OP intoxication includes pr
lactic and therapeutic use of derivatives
reversible ChE inhibitor physostigmine (es
[56]. Reactivators of AChE and BuChE we
signed to be attracted to the choline-bindin
(like choline, they possess a permanent po
charge) and to have a reactive group (–C=N
that can promote hydrolysis of the phosphat
blocks the enzyme's reactive site. A succe
reactivator is PAM (pyridinium-2 aldo
methiodide) [57]. This reactivation pro
though, is hampered by "ageing", which o
after ChEs interact with certain OPs, such as
(isopropylmethylphosphonofluoridate), DFP
sopropylphosphorofluoridate) and malat
(0,0-diethyldithiophosphate diethylmercapto
cinate). This process is believed to inv
dealkylation of the covalently bound OP gr
rendering therapy by oximes ineffective [3,
In addition to affecting the NMJ, OPs c
delayed damage to the central nervous sy
(CNS) [1], where the exact mechanism of
action is unknown. In all body tissues poiso
ChE inhibitors may be dealt with by pla
BuchE, which reacts with the agent, sparing
more vital AChE. Congenital variabilities
BuchE, however, may impair this protec
property.

BuChE levels in serum of individuals with
common BCHE allele have been shown to
with a standard deviation of 25% in the norm
non-exposed population [6]. In addition,
creased or decreased BuChE activities have b
attributed to some disease states. These incl
burn injuries, renal disease, or liver dysfuncti
for decrement of BuChE levels, whereas
creases have been observed in cases of obes
asthma, and alcoholism [27, 45, 55, 58]. This
turn, implies variable sensitivity of affected
dividuals to OP poisoning. It is therefore
surprising that long-term neurological and/or p
chiatric effects of OP poisoning in man have be
subject to dispute for many years. In rodents, t
OP agent tri-*o*-cresyl phosphate was shown to
duce chronic, progressive degeneration of lo
motor neurons [10] suggesting that parallel
fects may be anticipated in humans under occ
pational exposure. At least one such paraly
case has, indeed, been reported [17]. Subseque
reports of long-term neuropsychiatric distu
bances in individuals who were subject to chron
OP poisoning [16, 21] were reinforced by obse
vations of EMG abnormalities in occupational
exposed individuals [24]. In addition, acute pes

cide intoxication was reported to produce an abnormal EEG, similar to that detected in epileptic patients [11, 33] or other, less drastic yet long-term and persistent alterations [18, 51]. These conclusions are consistent with the hypothesis that cholinergic signaling is involved in memory [4] and calls for particular caution in the use of OP insecticides.

In contrast with these early and alarming studies, other investigators failed to observe long-term psychiatric [53] and/or neurological [12] changes following OP poisoning, either in man or in experimental animals. Neurobehavioral abnormalities were sought in workers chronically exposed to OP insecticides and no defects in performance could be observed in memory or language abilities [44]. A more recent epidemiological attempt to evaluate the latent neurological effects of OP pesticide poisoning was performed in Colorado and included 100 individuals with a history of previous acute OP pesticide poisoning, matched according to age and education with non-poisoned control individuals [46]. The study was performed several years after the exposures, and revealed no persistent alterations in audiometric, ophthalmic, EEG or blood ChE tests. However, neuropsychological abnormalities in memory, abstraction and mood as well as impaired motor reflexes were statistically significant in the OP-exposed individuals in the range characteristic of patients with cerebral damage or dysfunction. This study clearly demonstrates chronic neurological consequences of acute OP poisoning and explains the reasons for the previous disagreement between researchers, since it reveals sequelae too subtle to be detected by standard techniques.

A recent study further describes aggressive behavior following exposure to cholinesterase inhibitors [15]. This was reported in four different individuals with no previous history of violent behavior and appeared to be reversible, which adds unprovoked aggressive behavior to the list of neurotoxic effects of OP poisoning.

Thus, point mutations in the BCHE gene may cause congenital variability in the susceptibility of OP poisoning. Among the cholinergic drugs, ChE inhibitors used experimentally to treat senile patients, should be mentioned. They include mainly carbamates such as physostigmine and derivatives [54]. It is important to note that several of the variant BuChE subtypes drastically differ in their inhibition constants for carbamates [37]. In this case as well, phenotypic variabilities in the intensity and duration of response to cognitive drugs may be expected in individuals having the variant genotypes.

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References

1 Abou-Donia MB, Lapadula DM. Mechanisms of organophosphorous ester-induced delayed neurotoxicity: type I and type II. *Ann Rev Pharmacol Toxicol* 1990;30:405-40
2 Akasu T, Ariyoshi M, Tokimasa T. Postsynaptic modulation of cholinergic transmission by endogenous substances. *Comp Biochem Physiol* 1988;91C:241-6
3 Aldridge WN, Reiner E. *Enzyme Inhibitors as Substrates.* Amsterdam: North-Holland, 1972
4 Allain H, Moran P, Bentue-Ferrer D, Martinet JP, Lieury A. Pharmacology of the memory process. *Arch Gerontol Geriatr* (Suppl) 1989;1:109-20
5 Allderdice PW, Gardner HA, Galutira D, Lockridge O, La Du BN, McAlpine PJ. The cloned butyrylcholinesterase (BCHE) gene maps to a single chromosome site. 3q26, *Genomics* 1991;11:452-4
6 Altland K, Goedde HW, Held K, Jensen M, Munsch H, Solem E. New biochemical and immunological data on quantitative and qualitative variability of human pseudocholinesterase. *Humangenetik* 1971;14:56-60
7 Arpagaus M, Chatonnet A, Rogers L, Venta P, La Du, BN Lockridge O. Genomic clones for human butyrylcholinesterase. *J Cell Biol* 1989;107:521
8 Bartels CF, Zelinski T, Lockridge O. Mutation at codon 322 in the human AChE gene accounts for YT blood group polymorphism. *Am J Hum Genet* 1993;52:928-36
9 Bartels CF, Jensen FS, Lockridge O, van der Spek AFL, Rubinstein HM, Lubrano T, La Du BN. DNA mutation associated with the human butyrylcholinesterase K-Variant and its linkage to the atypical variant mutation and other polymorphic sites. *Am J Hum Genet* 1992;50:1086-1103
10 Bidstrup PL, Bonnell J, Beckett AG. Paralysis following poisoning by a new organic phosphorous insecticide (Mipafox). *Br Med J* 1953;1:1068-72
11 Brown HW. Electroencephalographic changes and disturbance of brain function following human organophosphate exposure. *Northwest Med* 1971;70:845-6
12 Clark G. Organophosphate insecticides and behavior: a review. *Aerosp Med* 1971;42:735-40
13 Coates PM, Simpson NE. Genetic variation in human erythrocyte acetylcholinesterase. *Science* 1972;175:1466-7
14 Costa LG. Interactions of neurotoxicants with neurotransmitter systems. *Toxicology* 1988;49:359-66

15 Devinsky O, Kernan J, Bear DM. Aggressive behavior following exposure to cholinesterase inhibitors. *J Neuropsychiatry Clin Neurosci* 1992;4:189-94

16 Dille JR, Smith PW. Central nervous system effects of chronic exposure to organophosphate insecticides. *Aerosp Med* 1964;35:475-

17 Drenth JH, Ensberg IFG, Roberts DV, Wilson A. Neuromuscular function in agricultural workers using pesticides. *Arch Environ Health* 1972;25:395-8

18 Duffy FH, Burchfield JL, Bartels PH, Gaon M, Sim VM. Long-term effects of an organophosphate upon the human electroencephalogram. *Toxicol Appl Pharmacol* 1979; 47:161-76

19 Durham WF, Hayes WJ Jr. Organic phosphorous poisoning and its therapy. *Arch Environ Health* 1962;5:21-47

20 Ehrlich G, Viegas-Pequignot V, Ginzberg D, Sindel L, Soreq H, Zakut H. Mapping the human acetylcholinesterase gene to chromosome 7q22 by fluorescent *in situ* hybridization coupled with selective PCR amplification from a somatic hybrid cell panel and chromosome-sorted DNA libraries. *Genomics* 1992;13:1192-7

21 Gershon S, Shaw FH. Psychiatric sequelae of chronic exposure to organophosphorous insecticides. *Lancet* 1961; i:1371-4

22 Getman DK, Eubanks JH, Camp S, Evans GA, Taylor P. The human gene encoding acetylcholinesterase is located on the long arm of chromosome 7. *Am J Hum Genet* 1992;51:170-177

23 Gnatt A, Prody CA, Zamir R, Lieman-Hurwitz J, Zakut H, Soreq H. Expression of alternatively terminated unusual human butyrylcholinesterase messenger RNA transcripts, mapping to chromosome 3q26-ter, in nervous system tumors. *Cancer Res* 1990;50:1983-7

24 Grob D, Harvey AM. The effects and treatment of nerve gas poisoning. *Am J Med* 1953;24:52-63

25 Harel M, Sussman JL, Krejci E, Bon S, Chanal P, Massoulie J, Silman I. Conversion of acetylcholinesterase to butyrylcholinesterase: modeling and mutagenesis. *Proc Natl Acad Sci USA* 1992;89:10827-31

26 Hodgkin WE, Giblett ER, Levine H, Bauer W, Motulsky AG. Complete pseudocholinesterase deficiency: genetic and immunologic characterization. *J Clin Invest* 1965; 44:486-97

27 Hunter D. *The Diseases of Occupations*. 5th Edn. London: English Universities Press, 1975;pp 368-77

28 Levene C, Bar-Shany S, Manny N, Moulds JJ, Cohen T. The Yt blood groups in Israeli Jews, Arabs, and Druse. *Transfusion* 1987;27:471-4

29 Lewis M, Kaita H, Philipps S, McAlpine PJ, Wong P, Giblett ER, Anderson J. The Yt blood group system (ISBT No. 011) Genetics studies. *Vox Sang* 1987;53:52-6

30 Lockridge O. Genetic variants of human serum cholinesterase influence metabolism of the muscle relaxant succinylcholine. *Pharmacol Ther* 1990;47:35-60

31 Lockridge O, Bartels CF, Vaughan TA, Won CK, Norton SE, Johnson LL. Complete amino acid sequence of human serum cholinesterase. *J Biol Chem* 1987;262:549-57

32 Loomis TA. Distribution and excretion of pyridine-2-aldoxime methiodide (PAM); atropine and PAM in sarin poisoning. *Toxicol Appl Pharmacol* 1963;5:489-99

33 Metcalf DR, Holmes JH. EEG, psychological, and neurological alteration in humans with organophosphorous exposure. *Ann NY Acad Sci* 1969;160:357-65

34 Michotte A, Van Dijck I, Maes V, D'Haenen H. Ataxia as the only delayed neurotoxic manifestation of organophosphate insecticide poisoning. *Eur Neurol* 1989;29:23-26

35 Murphy SD. Pesticides. *In:* Casarett LJ, Doull J, eds. *Toxicology – The Basic Science of Poisons*. New York: Macmillan, 1975

36 Neville LF, Gnatt A, Padan R, Seidman S, Soreq H. Anionic site interactions in human butyrylcholinesterase disrupted by two single point mutations. *J Biol Chem* 1990;265:20735-8

37 Neville LF, Gnatt A, Loewenstein Y, Seidman S, Ehrlich G, Soreq H. Intramolecular relationships in cholinesterases revealed by oocyte expression of site-directed and natural variants of human BCHE. *EMBO J* 1992;11:1641-9

38 Noguiera CP, McGuire MC, Graeser C *et al.* Identification of a frameshift mutation responsible for the silent phenotype of human serum cholinesterase, Gly 117 (GGT → GGAG). *Am J Hum Genet* 1990;46:934-42

39 Nogueira CP, Bartels CF, McGuire MC *et al.* Identification of two different point mutations associated with the fluoride-resistant phenotype for human butyrylcholinesterase. *Am J Hum Genet* 1992;51:821-8

40 Primo-Parmo SL, Bartels CF, Lightstone H, van der Spek AFL, La Du BN. Heterogeneity of the silent phenotype of human butyrylcholinesterase-identification of eight new mutations. *In:* Shaffeman A, Velan B, eds. *Multidisciplinary Approaches to Cholinesterase Functions*. New York: Plenum Press, 1992;pp 61-4

41 Prody C, Zevin-Sonkin D, Gnatt A, Koch R, Zisling R, Goldberg O, Soreq H. Use of synthetic oligodeoxynucleotide probes for the isolation of a human cholinesterase cDNA clone. *J Neurosci Res* 1986;16:25-35

42 Prody CA, Zevin-Sonkin D, Gnatt A, Goldberg O, Soreq H. Isolation and characterization of full-length clones coding for human cholinesterase from fetal human tissues. *Proc Natl Acad Sci USA* 1987;84:3555-9

43 Prody CA, Dreyfus PA, Zamir R, Zakut H, Soreq H. *De novo* amplification within a "silent" human cholinesterase gene in a family subjected to prolonged exposure to organophosphorous insecticides. *Proc Natl Acad Sci USA* 1989;86:690-4

44 Rodnitzky RL, Levin HS, Mick DL. Occupational exposure to organophosphate pesticides: neurobehavioral study. *Arch Environ Health* 1975;30:98-103

45 Rosalki SB. Genetic influences on diagnostic enzymes in plasma. *Enzyme* 1988;39:95-109

46 Savage EP, Keefe TJ, Mounce LM, Heaton RK, Lewis JA, Burcar PJ. Chronic neurological sequelase of acute organophosphate pesticide poisoning. *Arch Environ Health* 1988;43:38-45

47 Soreq H, Zakut H. *Human Cholinesterases and Anticholinesterases*. San Diego: Academic Press, 1993,324 pp

48 Soreq H, Ben-Aziz R, Prody CA *et al.* Molecular cloning and construction of the coding region for human acetylcholinesterase reveals a G+C-rich attenuating structure. *Proc Natl Acad Sci USA* 1990;87:9688-92

49 Soreq H, Gnatt A, Loewenstein Y, Neville LF. Excavations into the active-site gorge of cholinesterases. *Trends Biochem Sci* 1992;17:353-8

50 Spring FA, Anstee DJ. Evidence that the antigens of the Yt blood-group system are located on human erythrocyte acetylcholinesterase. *Blood* 1992;80:2136-41

51 Stoller A, Krupinski J, Christophers AJ, Blanks GK: Organophosphorous insecticides and major mental illness: an epidemological investigation. *Lancet* 1965;i:1387-8

52 Sussman JL, Harel M, Frolow F, Oefner C, Goldman A, Toker L, Silman I. Atomic structure of acetylcholinesterase from *Torpedo californica:* a prototypic acetylcholine-binding protein. *Science* 1991: 253:872-9

53 Tabershaw IR, Cooper WC. Sequelae of acute organic phosphate poisoning. *J Occup Med* 1966;8:5-20

54 Taylor P. Cholinergic agonists, Cholinergic antagonists, Agents acting at the neuromuscular junction and autonomic ganglia. *In:* Gilman AG, Rall TW, Nies AS, Taylor P, eds. *Goodman and Gilman's The Pharmacological Basis of Therapeutics.* 8th edition. New York: Pergamon Press, 1990:pp 122-130, 131-149, 166-186

55 Whittaker M. *Cholinesterase.* Basel:Karger, 1986

56 Wills JH. Toxicity of anticholinesterases and treatment of poisoning. *In: International Encyclopedia of Pharmacology and Therapeutics.* Vol. I, Oxford: Pergamon Press, 1970:pp 357-69

57 Wilson IB. Molecular complementarity and antidotes for alkylphosphate poisoning. *Fed Proc* 1959;18:752-8

58 Zakut H, Even L, Birkenfeld S, Malinger G, Zisling R, Soreq H. Modified properties of serum cholinesterases in primary carcinomas. *Cancer* 1988;61:727-37

TRANSGENIC EXPRESSION OF HUMAN ACETYLCHOLINESTERASE INDUCES PROGRESSIVE COGNITIVE DETERIORATION IN MICE

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Running Head: Cognitive damage in AChE transgenic mice

Abstract

Background : The cognitive deterioration characteristic of Alzheimer's disease is notably associated with structural changes and subsequent cell death which primarily occur in acetylcholine producing neurons, progressively damaging cholinergic neurotransmission. We have previously reported that excess acetylcholinesterase (AChE), leading to synaptic cholinergic imbalance, alters structural features of neuromuscular junctions in transgenic *Xenopus* tadpoles. However, the potential of cholinergic imbalance to induce progressive decline of memory and learning in mammals has not been explored.

Results : To approach the molecular mechanisms underlying the progressive memory deficiencies associated with impaired cholinergic neurotransmission, we created transgenic mice expressing human AChE in brain neurons. With enzyme levels up to two-fold higher than in controls, these mice displayed age-independent resistance to the hypothermic effects of the anti-AChE paraoxon. However, in addition to this improved scavenging capacity for anti-AChEs these transgenic mice also resisted muscarinic, nicotinic and serotonergic agonists, indicating secondary pharmacological changes. Moreover, the ACHE-transgenic mice developed progressive learning and memory impairments, although their locomotor activities and open field behavior remained similar to those of matched control mice. Within 6 months of age, these mice totally lost their ability to respond to training in a spatial learning water maze test, in contradistinction with their normal performance in this test at the age of 4 weeks. This animal model is hence suitable for investigating the transcriptional changes associated with cognitive deterioration and for testing potential drugs for attenuating such progressive damages.

Conclusion: We conclude that cholinergic imbalance may by itself cause progressive memory decline in mammals, suggesting that congenital and/or acquired changes in this vulnerable balance may contribute toward the physiopathology of Alzheimer's disease.

Background

Progressive deterioration of memory and learning is a characteristic manifestation of Alzheimer's disease [1]. The molecular mechanisms underlying this phenomenon are still obscure, as the vast majority (>90%) of Alzheimer's disease cases is of sporadic origin, with presumably multigenic contributions combined with environmental causes [2]. This calls for searching for potential reasons to explain the progressive cognitive decline in Alzheimer's disease.

At the neuropathological level, the neuritic plaques and neurofibrillary tangles which are defining features of this disease are particularly concentrated in brain regions where cholinergic circuits operate [3]. Moreover, the progression of disease symptoms is primarily associated with structural changes in cholinergic synapses [4], loss of particular acetylcholine receptor subtypes [5], death of acetylcholine (ACh) producing neurons [6] and consequent damage to cholinergic neurotransmission [7], leaving a relative excess of the ACh hydrolyzing enzyme acetylcholinesterase (AChE). This spatiotemporal correlation led to the cholinergic theory of Alzheimer's disease [7] and promoted development of cholinergic therapies for it [8]. The first of these drugs to be approved for clinical use is tacrine (THA, tetrahydroaminoacridine, Cognex®), an AChE inhibitor which temporarily attenuates the worsening of certain clinical symptoms in patients at the early stages of Alzheimer's disease [8]. The concept behind the use of tacrine is to restore the cholinergic balance, at least for a while, by elevating ACh levels and augmenting the functioning of remaining receptors [9]. The finding that tacrine administration has some therapeutic value emphasizes the importance of balanced cholinergic neurotransmission for satisfactory steady-state cognitive functioning. However, the question whether imbalanced cholinergic neurotransmission can by itself contribute toward the progressive decline in Alzheimer's disease has not been addressed.

To examine the *in vivo* consequences of synaptic cholinergic imbalance, we have recently expressed human AChE in transiently transgenic embryos of *Xenopus laevis* frogs [10]. The recombinant human enzyme accumulated in neuromuscular junctions and altered their structural features [11]. Moreover, transgenic expression of mouse muscle nicotinic ACh receptor conferred similar structural changes onto *Xenopus* neuromuscular junctions, enlarging their post-synaptic length and deepening their synaptic cleft [11]. These structural alterations resembled those reported for cholinergic brain synapses in patients at the early stages of Alzheimer's disease [4], suggesting that the latter changes as well could perhaps be caused by imbalanced cholinergic neurotransmission. This, in turn, initiated our interest in creating transgenic mice with congenital AChE overexpression and examining cognitive functions in these animals.

The construction of our transgenic mice was conceptually different from other recent models designed to recreate the neuropathological features of Alzheimer's disease. Thus, transgenic mice expressing normal or mutant forms of the ß-amyloid protein died prematurely [12] or developed amyloid plaques rich in ß-amyloid peptides [13]. This shed new light on the histopathological changes characteristic of Alzheimer's disease, particularly in carriers of ß-amyloid mutations, but not on the relevant mental deficiencies. In contrast, eliminating several key brain proteins by homologous recombination (knockout) caused no histopathological changes, but damaged spatial learning [14-16] or avoidance learning [17]. However, neither the ß-amyloid transgenics nor any of these knockout mice were reported to display the neurochemical changes particular to Alzheimer's disease, one of them being cholinergic imbalance. Moreover, none of the reported cognitive changes seemed to involve progressive deterioration.

In contrast with the above summarized studies, our efforts were devoted toward creating a subtle change in the normal balance of cholinergic neurotransmission within the mammalian brain, mimicking conditions which might exist in patients with no hereditary tendency for Alzheimer's disease. We further limited this change to those cell types in which it naturally takes place and studied its neurochemical, physiopathological and cognitive consequences in an age-dependent manner. Our findings demonstrate that AChE overexpressing mice with inheritable cholinergic imbalance undergo selective progressive decline in their capacity for spatial learning and memory and suggest the use of these mice for exploring the detailed molecular mechanisms underlying the cognitive deterioration in Alzheimer's disease.

Results

Low level AChE overexpression is compatible with life: To elicit transgenic mice overexpressing AChE, we employed DNA encoding the brain and muscle form of human AChE [10], preceded by the potent ubiquitous promoter of cytomegalovirus (CMV) [18]. Alternatively, we used a 596 nucleotide long fragment from the authentic promoter of the human ACHE gene, followed by its first intron and the same coding sequence (HpACHE; [10]). Both promoters were previously found to direct human AChE accumulation in *Xenopus* neuromuscular junctions [10,11], although the CMV promoter was ca. 20-fold more efficient in its capacity to direct production of the AChE protein in *Xenopus* [10]. The outcome of our mouse experiments was rather different: out of 70 microinjections of CMVACHE DNA into mouse eggs, we obtained only one viable founder mouse carrying the CMVACHE transgene. Moreover, the transgenic DNA was not expressed in the progeny of that mouse. Parallel 40 microinjections of HpACHE DNA yielded 3 viable pedigrees with 2, 10 and 15 copies of the transgene. Of these, no enzyme production was observed in the two pedigrees with higher copy numbers of the transgene. Transgenic DNA remained expressible only in that pedigree with the lowest amount (2 copies) of HpACHE DNA. These exceedingly low success rates in obtaining viable AChE-transgenic mice were considerably lower than those we previously observed with other DNAs [19,20], suggesting that AChE overexpression was compatible with life only at low levels. Seven generations of transgenic mice with unmodified HpACHE DNA were thereafter raised from the above pedigree, all of which presented grossly normal development, activity and behavior.

Transgenic HpACHE expression is limited to CNS neurons: Reverse transcription and quantitative PCR amplification [20] revealed both human and mouse ACHEmRNA transcripts in dissected brain regions of the apparently homozygous transgenic mice from the fifth and subsequent generations, as is

demonstrated in Fig. 1A. Host ACHEmRNA levels and alternative splicing patterns [21] remained apparently unchanged. Bone marrow and adrenal glands displayed only mouse ACHEmRNAs, with apparently unmodified concentrations of the principal alternative products. In spite of several myoD elements in the HpACHE promoter [10], the transgene was not expressed in muscle .

To associate human ACHEmRNA transcripts with specific brain cell types, we performed *in situ* hybridization experiments. Labeling was seen in essentially similar brain neurons as those observed in other mammals [22,23], including cell bodies in the basal forebrain, brainstem and cortex of transgenic mice. Cholinoceptive hippocampal neurons were intensively labeled, especially in the CA1-CA2 region (Fig. 1B). Thus, expression of the HpACHE transgene was apparently confined to host central nervous system (CNS) neurons that normally express the mouse ACHE gene.

Normally processed transgenic AChE accumulates in cholinergic brain regions: AChE from the brain of control and transgenic mice displayed similar sedimentation profiles in sucrose gradient centrifugation, demonstrating unmodified assembly into multimeric enzyme forms (Fig. 1C). Up to 50% of the active enzyme in basal forebrain (Fig. 1C), but none in bone marrow (Table I), interacted with monoclonal antibodies specific to human AChE [24]. Catalytic activity measurements in tissue homogenates revealed a 100% increase over control in the detergent-extractable amphiphilic AChE fraction from basal forebrain and more limited increments in cortex, brainstem, cerebellum and spinal cord extracts (Table I). There were no age-dependent changes in this pattern and no differences in the cell type composition of bone marrow of transgenic as compared with control mice. Gel electrophoresis followed by cytochemical staining of enzyme activity [24] revealed similar migration for AChE from the brain of transgenic and control mice, indicating comparable glycosylation patterns.

Intensified cytochemical staining of AChE activity was observed in brain sections from transgenic mice in all of the areas that stain for AChE activity in normal mice [25]. Staining was particularly intense in the neo-striatum and pallidum (Fig. 2A) as well as in hippocampus (Fig. 2B), the latter two areas associated with learning and memory [26,27]. Moreover, there were more conspicuous depositions of the electron-dense product of AChE cytochemical staining within synapse-forming dendrites in the anterior hypothalamus from transgenic mice than in control brain sections (Table II). However, synapses interacting with these stained dendrites were of similar length in transgenic mice and in controls (Table II), demonstrating that unlike the situation in *Xenopus laevis* neuromuscular junctions [11,24], AChE overexpression in the mouse brain did not modify the post-synaptic length of at least part of the cholinergic synapses. In addition, there was no sign for neurofibrillary tangles or amyloid plaques in the analyzed brain sections from mice up to 6 months of age.

Transgenic AChE selectively alters hypothermic drug responses: In search for the physiological involvement of transgenic AChE, and since cholinergic synapses in the anterior hypothalamus, where we noted AChE overexpression, are involved in thermoregulation [28], we examined hypothermic responses of these transgenic mice to the potent AChE inhibitor diethyl p-nitrophenyl phosphate (paraoxon), the toxic metabolite of the agricultural insecticide parathion. Both young (4 to 5 weeks old) and adult (5 to 7 months old) transgenic mice were considerably more resistant to paraoxon-induced hypothermia than non-transgenic controls (Fig. 3A, B). The transgenic mice were almost totally resistant to a low paraoxon dose (0.25 mg/kg, Fig. 3A). With higher doses, they displayed limited reduction in body temperature and shorter duration of response as compared with controls. Most importantly, transgenic mice exposed to 1 mg/kg dose of paraoxon (Fig. 3B) retained apparently normal physical activity, while control mice experienced

under this dose myoclonia and muscle stifening, symptoms characteristic of cholinergic overstimulation.

In addition to their improved capacity for scavenging of the anti-AChE paraoxon, the transgenic mice also displayed resistance to the hypothermic effects of the muscarinic agonist oxotremorine (Fig. 3C), to the less potent effect of nicotine (Fig. 3D), and to the serotonergic agonist 8-hydroxy-2-(di-*n*-propylamino) tetralin (8-OH-DPAT) (Fig. 3E), but not to the α_2-adrenergic agonist clonidine (Fig. 3F). Also, thermal response to cold exposure remained unchanged in the transgenic compared to the control mice: within 60 min exposure to 5 °C, body temperature decreased to 35.5 °C in both, demonstrating that no changes had occurred in their peripherally-induced control over body temperature.

Transgenic AChE induces age-dependent decline in spatial learning capacity: To examine their cognitive functioning, transgenic and control mice were subjected to several behavioral tests. When compared to sex-matched groups of non-transgenic control mice at the age of 1, 2-3 and 5-7 months, the AChE-transgenic mice retained normal locomotor and explorative behavior, covering the same space and distance in an open field as their control counterparts. At the same time the open field anxiety of these mice remained unchanged, as evaluated in the frequency of defecation incidents and grooming behavior (Table III). In contrast, conspicuous behavioral differences were observed in these mice in two versions of the Morris water maze [29]. In the hidden platform version of this test mice are expected to use long-distance cues to orient themselves, find a platform submerged under opaque water and escape a swimming task [29]. The transgenics' performance in this test was apparently normal at the young age of 4 weeks, when they needed a similar number of training sessions as controls to reach the platform within significantly shorter time (defined as the escape latency) than untrained mice (Fig 4A and Table III). At the age of 2-3 months, transgenics already needed 8

more training sessions than controls to improve their performance. In addition, their inter-animal variability increased and their shortest escape latency was significantly longer than that of control mice of the same age (Fig 4B and Table III). Finally, at the age of 6 months, naive transgenic mice, but not controls, became totally incapable of improving their performance in this test through training, so that their escape latency remained the same as that of untrained mice (Fig 4C and Table III). This deterioration pattern was not caused by locomotion deficiencies, as was clear when the two groups of mice were compared by the open field test (Table III). Moreover, this progressive decline in spatial learning and memory was more pronounced than those observed in mice with knocked-out glutamate receptor 1 [14], fyn [15] or calcium calmoduline kinase II [16], suggesting a more substantial perturbation than in any of these defects.

A different, age-independent defect was observed in the visible platform version of the Morris water maze, in which mice are trained to escape the swimming task by using short-distance cues to climb on a visible platform decorated by a flag and a paper cone [29]. Interestingly, the transgenics could not improve their performance in this test through training, regardless of their age (Table III), perhaps due to early-onset difficulties in short-distant vision or because of abnormal avoidance behavior. In either case, the early occurrence of this defect at the age of four weeks, when the hidden platform test was still correctly performed by these transgenic mice, demonstrates that the defects in the visual and the hidden platform test were unrelated.

Discussion

Using the authentic promoter from the human ACHE gene in conjunction with the AChE coding sequence, we created transgenic mice expressing human AChE in CNS neurons in levels which were apparently low enough to be compatible with life. The transgenic enzyme was normally processed and was detected preferentially in brain areas normally expressing AChE, where it affected transgenic animal responses to drugs causing hypothermia. Most importantly, rapid decline in the spatial learning capacities of these transgenic animals initiated in early adulthood and progressed thereafter, supporting the notion that subtle cholinergic imbalance can cause progressive learning deficiencies in mammals.

Our failure to obtain viable mice with high levels of AChE overexpression may imply that both the HpACHE promoter with its limited efficacy and the low copy number of the transgene were important for allowing survival of these AChE overexpressing mice. The expression pattern of the transgene excluded peripheral organs where the ACHE gene is normally expressed [30-32]. Moreover, there was no expression in muscle, in spite of MyoD elements included in the transgene. This restriction to CNS neurons expressing AChE may be due to an incomplete promoter, missing necessary enhancers. Alternatively, silencing elements in the employed promoter, like those reported in upstream sequences of the choline acetyltransferase gene [33], could prevent expression of this transgene in its normal target tissues. Finally, the insertion site within the host genome may affect expression. Additional studies will be required to distinguish between these possibilities.

The limited neuronal expression and the low copy number of the transgene ensured that it could only cause subtle cholinergic imbalance. Thanks to the extremely high homology (95%) between the human and the mouse enzymes [30-32], the transgenic protein was processed and assembled normally. It

therefore accumulated in all of the relevant sites, with its highest excess levels found in basal forebrain, the region most vulnerable to neuronal loss in Alzheimer's disease [1]. Interestingly, cholinergic synapses within the brain of these transgenic mice retained their normal length, unlike neuromuscular junctions from AChE-overexpressing *Xenopus* tadpoles. However, the *Xenopus* synapses accumulated up to 10-fold more AChE [11,24]. In contrast, enzyme excess was limited to 2-fold in those mouse synapses which were analysed, which perhaps reflects the top level compatible with survival. The question whether cholinergic imbalance can cause progressive structural changes to other particular types of cholinergic synapses in the mammalian brain must await further measurements.

The resistance of these transgenic mice to the induction of hypothermia by different agents demonstrated modified functioning of cholinergic synapses overexpressing human AChE. Resistance to paraoxon could be expected, as the amount of its target protein, AChE, was significantly increased in the brain of these mice. However, the resistance to muscarinic, nicotinic and serotonergic agonists reflected secondary changes affecting both cholinergic and serotonergic synapses. These changes were caused, either directly or indirectly, by the transgenic enzyme. Yet, no general alterations occurred in the network of neurons controlling thermoregulation, as these transgenic mice retained normal responses to noradrenergic agents and to cold exposure. This suggests loss of specific synapses and/or receptor desensitization as possible causes and indicates the use of cholinergic and serotonergic agonists for early diagnosis of imbalanced cholinergic neurotransmission in human patients.

From their young adulthood onward, and also at a time when their spatial learning performance with a hidden platform was indistinguishable from that of controls, our transgenic mice failed to respond to short-distance visible cues. This can reflect a particular avoidance behavior. Alternatively, it can be due to the inability of these mice to focus their eyesight on a nearby target.

This calls to mind the differential response of Alzheimer's disease patients to tropicamide [34]. It is possible that the autonomic control over pupil constriction and eyesight-focusing muscles requires precisely balanced cholinergic neurotransmission. It will be intriguing to examine whether Alzheimer's disease patients have more difficulties in focusing on nearby targets than normal aged patients.

Of particular interest are the gradual spatial memory deficits displayed by the AChE-overexpressing transgenic mice in the hidden platform water maze, as opposed to their normal open field behavior. The early adulthood onset of these impairments suggests that the cholinergic balance essential for spatial memory can be sustained in the young transgenic mice, perhaps by a feedback mechanism adjusting other key elements in cholinergic synapses. This intricate process resembles the increase in α-bungarotoxin binding levels in AChE-overexpressing *Xenopus* embryos and the reciprocal AChE accumulation in neuromuscular junctions of tadpoles expressing the mouse nicotinic ACh receptor [11]. In extension of those observations, we now learn that in mammals this adjustment of the cholinergic balance can only be maintained for a limited time.

Several multileveled mechanisms are known which can be involved in controlling CNS cholinergic balance. Various ACh receptor genes were shown to share common promoter elements [35,36]. This ensures co-regulation through trans-acting factors. Also, a single cis-acting promoter controls the production of both the vesicular ACh transporter and choline acetyltransferase [37,38]. Gradual failure to maintain either or both of these mechanisms, or others, may be involved in the progressive changes observed in our mice. Moreover, the appearance of memory perturbation in the early stages of Alzheimer's disease may similarly signal the onset of failure to sustain cholinergic balance at the precision levels required for memory. The increase in brain AChE activity under stress [39] and the heterogeneous genomic origin

of Alzheimer's disease in the vast majority of patients [2] may indicate that failure to maintain cholinergic balance can be initiated due to variable causes.

Whereas the phenotypic differences observed in these transgenic mice are most probably correlated with ACh hydrolysis, further experiments should be performed to explore correlation with the potential non-catalytic functions of AChE, e.g. in cell-cell interactions [40,41]. In either case, the gradual impairment of learning and memory due to AChE overexpression is consistent with the temporary partial improvement when anti-AChE drugs are administered to Alzheimer's disease patients [8,9]. Moreover, preliminary differential PCR displays [42,43] of mRNA from dissected brain regions of our transgenic mice already revealed age-dependent transcriptional changes, which await characterization. This approach can unravel the target genes under control. Testing the effects of drug treatments on the progressive transcriptional changes occurring in the brain of these mice can thereafter lead to development of novel strategies for prophylactic treatment of neurodegenerative disorders associated with cholinergic imbalance.

Finally, while AChE-overexpressing mice provide a model for behavioral, physiological and molecular aspects of cholinergic imbalance in mammals, they also demonstrate a dissociation between this imbalance and the ß-amyloid deposits occurring in Alzheimer's disease. The possibility remains that cholinergic neurons are particularly sensitive to the neurotoxicity attributed to the ß-amyloid peptides [12,13], in which case the ß-amyloid transgenic mice should develop cholinergic deficiencies with time. Alternatively, or in addition, cholinergic imbalance may lead to abnormal ß-amyloid expression, in which case the AChE transgenic mice should develop ß-amyloid deposits with time. A third possibility is that the linkage between the pathophysiological and the cognitive manifestations in Alzheimer's disease is unique to *Homo sapiens*.

Conclusions

Expression of human AChE in CNS neurons of transgenic mice created spatial learning and memory impairments which appeared in these mice shortly after early adulthood and worsened progressively thereafter. In contrast, the open field behavior of these transgenic animals remained normal, while their responses to hypothermia-inducing drugs were decreased and their performance in a visible water maze test was perturbed in a stable manner, from early adulthood onward. These findings suggest that subtle alterations in the cholinergic balance may cause physiologically-observable changes and contribute to memory deterioration in at least part of the patients with sporadic Alzheimer's disease.

Materials and methods

Transgenic mice production: Noted DNAs were injected into fertilized eggs of FZB/N mice as previously described [19,20]. PCR amplification and blot hybridization of tail DNA samples verified integration of variable copy numbers of the transgene into the host genome of founder mice and their progeny.

RNA extraction and PCR amplification: These were performed as detailed elsewhere [20], except that the annealing temperature was 69 °C. Species-specific PCR primers were designed for human ACHEmRNA at nucleotides 1522 (+) and 1797 (-) in exons 4 and 6 [24] and for mouse, at nucleotides 375 (+) and 1160 (-) in exons 2 and 3 [21]. Resultant PCR products (275 and 785 bp, respectively) were electrophoresed on agarose gels.

***In situ* hybridization**: Wholemount *in situ* hybridizations were performed at 65 °C on 50 µm thick 4% paraformaldehyde/ 1% glutaraldehyde-fixed sections of transgenic and control brains with digoxigenin-labeled complementary human ACHEcRNA. Detection was with alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer/ Mannheim).

AChE activity measurements: Acetylthiocholine hydrolysis levels were determined following sucrose gradient centrifugation of tissue homogenates prior to or following adhesion to human-selective anti-AChE monoclonal antibodies, essentially as detailed elsewhere [24,32].

AChE cytochemical staining: Wholemount cytochemical staining of AChE activity was performed on 50 µm paraformaldehyde-glutaraldehyde fixed sections of brains essentially as detailed [24], except that incubation was for 2 h. Electron microscopy was thereafter performed on 80 nm sections of paraformaldehyde-glutaraldehyde fixed brain [24]. Control experiments, with no acetylthiocholine verified that these electron-dense deposits were indeed reaction products of *in situ* AChE catalysis.

Hypothermic response measurements: Core body temperature was measured rectally in mice with a thermocoupled element of 1 mm diameter

and 10 mm length at the noted times after intraperitoneal injections of paraoxon, oxotremorine, nicotine, 8-OH-DPAT or clonidine.

Morris water maze tests: Mice were tested in a 60 x 60 x 15 cm water maze filled with 1.5 mg/l powdered milk, with a submerged hidden platform in a fixed location 1 cm below water level. Four trials of up to 2 min were performed per day for 4 days, each initiated randomly in a different corner of the maze. For the visual platform test, water level was lowered by 2 cm and a 10 cm high striped flag and a 7 cm high black cone were positioned on top of the now visible platform. Mean escape latencies per day (4 sessions) were calculated for transgenic and sex- and age- matched controls, and statistically significant reductions of latencies were searched for as compared with the first day values in each section (one way ANOVA, followed by Neuman Keuls test).

Open field test: Mice were placed in one corner of a 60 x 60 cm Plexiglas box with 30 cm high walls and floor divided into 144 squares of 5X5 cm each. Using these squares, the motion path of the animals was manually traced for 5 minutes, which enabled measurements of walking distance and explored area. Grooming behavior and incidence of defecations were also noted.

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References

1. Katzman R.: Alzheimer's disease. N Engl J Med 1986, **314**: 962-973.
2. Ashall F. and Goate A. M.: Role of the β-amyloid precursor protein in Alzheimer's disease. Trends Biochem Sci 1994, **19**: 42-46.
3. Yankner B. A. and Mesulam M. M.: Seminars in medicine of the Beth Israel Hospital, Boston. Beta-Amyloid and the pathogenesis of Alzheimer's disease. N Engl J Med 1991, **325**: 1849-1857.
4. DeKosky S. T. and Scheff S. W.: Synapse loss in frontal cortex biopsies in Alzheimer's disease: correlation with cognitive severity. Ann Neurol 1990, **27**: 457-464.
5. Mash D. C., Flynn D. D. and Potter L. T.: Loss of M2 muscarinic receptors in the cerebral cortex in Alzheimer's disease and experimental cholinergic denervation. Science 1985, **228**, 1115-1117.
6. Davies P. and Maloney A. J.: Selective loss of central cholinergic neurons in Alzheimer's disease. Lancet 1976, **2**: 1403.
7. Coyle J. T., Price D. L. and DeLong M. R.: Alzheimer's disease: a disorder of cortical cholinergic innervation. Science 1983, **219**, 1186-1189.
8. Davies R. E., Emmerling M. R., Jaen J. C., Moos W. H. and Spiegel K.: Therapeutic intervention in dementia. Clinical Reviews in Neurobiology 1993, **7**: 41-83.
9. Knapp M. J., Knopman D. S., Solomon P. R., Pendlebury W. W., Davis C. S. and Gracon S. I: A 30-week randomized controlled trial of high-dose tacrine in patients with Alzheimer's disease. J Am Med Assn 1994, **271**: 985-991.
10. Ben Aziz-Aloya R., Seidman S., Timberg R., Sternfeld M., Zakut H. and Soreq H.: Expression of a human acetylcholinesterase promoter-reporter construct in developing neuromuscular junctions of Xenopus embryos. Proc Natl Acad Sci USA 1993, **90**: 2471-2475.
11. Shapira M., Seidman S., Sternfeld M., Timberg R., Kaufer D., Patrick J. and Soreq H.: Transgenic engineering of neuromuscular junctions in

Xenopus laevis embryos transiently overexpressing key cholinergic proteins. Proc Natl Acad Sci USA 1994, **91**: 9072-9076.

12. LaFerla F. M., Tinkle B. T., Bieberich C. J., Haudenschield C. C. and Jay G.: The Alzheimer's Aβ peptide induces neurodegeneration and apoptotic cell death in transgenic mice. Nature Genet 1995, **9**: 21-31.
13. Games D., Adams D., Alessandrini R., Barbour R., Berthelette P., Blackwell C. *et al.*: Alzheimer-type neuropathology in transgenic mice overexpressing V717F β-amyloid precursor protein. Nature 1995, **373**: 523-527.
14. Conquet F., Bashir Z. I., Davies C. H., Daniel H., Ferraguti F., Bordi F. *et al.*: Motor deficit and impairment of synaptic plasticity in mice lacking mGluR1. Nature 1994, **372**: 237-243.
15. Grant S. G. N., O'Dell T. J., Karl K. A., Stein P. L., Soriano P. and Kandel E. R.: Impaired long-term potentiation, spatial learning, and hippocampal development in fyn mutant mice. Science 1992, **258**: 1903-1909.
16. Silva A. J., Paylor R., Wehner J. M. and Tonegawa S.: Impaired spatial learning in α–calcium-calmodulin kinase II mutant mice. Science 1992, **257**: 206-211.
17. Picciotto M. R., Zoli M., Léna C., Bessis A., Lallemand Y., LeNovère N. *et al.*: Abnormal avoidance learning in mice lacking functional high-affinity nicotine receptor in the brain. Nature 1995, **374**: 65-67.
18. Schmitt E. V., Christoph G., Zeller R. and Leder P.: The cytomegalovirus enhancer: a pan-active control element in transgenic mice. Mol Cell Biol 1990, **10**: 4406-4411.
19. Shani M.: Tissue-specific expression of rat myosin light-chain 2 gene in transgenic mice. Nature 1985, **314**: 283-286.
20. Beeri R., Gnatt A., Lapidot-Lifson Y., Ginzberg D., Shani M., Soreq H. and Zakut H.: Testicular amplification and impaired transmission of human butyrylcholinesterase cDNA in transgenic mice. Human Reprod. 1994, **9**: 284-292.

21. Rachinsky T. L., Camp S., Li Y., Ekström T. J., Newton M. and Taylor P.: Molecular cloning of mouse acetylcholinesterase: tissue distribution of alternatively spliced mRNA species. Neuron 1990, **5**: 317-327.

22. Landwehrmeyer B., Probst A., Palacios J. M. and Mengod G.: Expression of acetylcholinesterase messenger RNA in human brain: an in situ hybridization study. Neuroscience 1993, **57**: 615-634.

23. Hammond P., Rao R., Koenigsberger C. and S. Brimijoin.: Regional variation in expression of acetylcholinesterase mRNA in adult rat brain analyzed by in situ hybridization. Proc Natl Acad Sci USA 1994, **91**: 10933-10937.

24. Seidman S., Sternfeld M., Ben Aziz-Aloya R., Timberg R., Kaufer-Nachum D. and Soreq H.: Synaptic and epidermal accumulations of human acetylcholinesterase are encoded by alternative 3'-terminal exons. Mol Cell Biol 1995, in press.

25. Kitt C. A., Höhmann C., Coyle J. T. and Price D. L.: Cholinergic innervation of mouse brain structures. The Journal of Comparative Neurology 1994, **341**, 117-129.

26. Yu J., Thomson R., Huestis P. W., Bjelajac V. M. and Crinella F. M.: Learning ability in young rats with single and double lesions to the "general learning system". Physiol Behav 1989, **45**: 133-144.

27. Eichenbaum H., Steward C. and Morris R. G. M.: Hippocampal representation in place learning. J. Neurosci. 1990, **10**: 3531-3542.

28. Simpson C. V., Ruwe W. D. and Myers R. D.: Prostaglandins and hypothalamic neurotransmitter receptors involved in hypothermia: a critical evaluation. Neurosci. Behavior. Rev. 1994, **18**: 1-20.

29. Morris R. G. M., Garrud P., Rawlins J. N. P. and O'Keefe J.: Place navigation impaired in rats with hippocampal lesions. Nature 1981, **297**: 681-682.

30. Massoulié J., Pezzementi L., Bon S., Krejci E. and Valette F.M.: Molecular and cellular biology of cholinesterases. Prog Neurobiol 1993, **41**: 31-91.

31. Taylor P. and Radic Z.: The cholinesterases: from genes to proteins. Ann Rev Pharmacol Toxicol 1994, **34**: 281-320.

32. Soreq H. and Zakut H.: Human Cholinesterases and Anticholinesterases. Academic Press, San Diego, 1993.

33. Li Y.P., Baskin F., Davis R. and Hersch L.: Cholinergic neuron-specific expression of the human choline acetyltransferase gene is controlled by silencer elements. J Neurochem 1993, **61**: 748-751.

34. Scinto L. F. M., Daffner K. R., Dressler D., Ransil B. I., Rentz D., Weintraub S. *et al.*: A potential noninvasive neurobiological test for Alzheimer's disease. Science 1994, **266**: 1051-1054.

35. Jia H. T., Tsay H. J. and Schmitt J.: Analysis of binding and activating functions of the chick muscle acetylcholine receptor γ-subunit upstream sequence. Cell Mol Neurobiol 1992, **12**: 241-258.

36. Laufer R. and Changeux J.P.: Activity-dependent regulation of gene expression in muscle and neuronal cells. Mol Neurobiol 1989, **3**: 1-53.

37. Erickson J. D., Varoqui H., Schäfer M. K. H., Modi W., Diebler M.-F., Weihe E. *et al.*: Functional identification of a vesicular acetylcholine transporter and its expression from a "cholinergic" gene locus. J Biol Chem 1994, **269**: 21929-21932.

38. Bejanin S., Cervini R., Mallet J. and Berrard S: A unique gene organization for two cholinergic markers, choline acetyltransferase and a putative vesicular transporter of acetylcholine. J Biol Chem 1994, **269**, 21944-21947.

39. Tsakiris S. and Kontopoulos A. N.: Time changes in Na+,K(+)-ATPase, Mg(++)-ATPase, and acetylcholinesterase activities in the rat cerebrum and cerebellum caused by stress. Pharmacology, Biochemistry and Behavior 1993, **44**: 339-342.

40. Layer P. G., Weikert T. and Alber R.: Cholinesterases regulate neurite growth of chick nerve cells in vitro by means of a non-enzymatic mechanism. Cell Tissue Res 1993, **273**: 219-226.

41. Small D. H., Reed G., Whitefield B. and Nurcombe V.: Cholinergic regulation of neurite outgrowth from isolated chick sympathetic neurons in culture. J Neurosci 1995, **15**: 144-151.

42. Liang P. and Pardee A. B.: Differential display of eukayotic messenger RNA by means of the polymerase chain reaction. Science 1992, **257:** 967-971.

43. Lev-Lehman E., El-Tamer A., Yaron A., Grifman M., Ginzberg D., Hanin I. and Soreq M.: Cholinotoxic effects on acetylcholinesterase gene expression are associated with brain-region specific alterations in G,C-rich transcripts. Brain Res 1994, **661**: 75-82.

LEGENDS TO FIGURES

Fig. 1 Detection of transgenic ACHEmRNA and active enzyme.

(A) **Identification of human ACHEmRNA**. RNA was extracted and amplified from transgenic (+) and control (-) cortex (CO), brainstem and cerebellum (BC), basal forebrain (BF) and bone marrow (BM). Species-specific PCR primers were designed for the indicated positions on the schemes above. Resultant PCR products (275 and 785 bp, respectively) were electrophoresed on agarose gels. Note that expression of HpAChE mRNA was limited to the central nervous system.

(B) ***In situ*** **hybridization**. Wholemount *in situ* hybridizations were performed on fixed brain sections from transgenic and control mice. CA1 hippocampal neurons (HIPPO-CA1) in the transgenic, but not the control section were intensely labeled.

(C) **Overexpressed AChE activity.** Acetylthiocholine hydrolysis levels were determined for tissue homogenates from control (C) and transgenic (T) mice after sucrose gradient centrifigation prior to (clear areas) or following adhesion to human-selective anti-AChE monoclonal antibodies (shaded area). Vertical arrows denote sedimentation of an internal marker, bovine catalase (11.4 S).

Fig. 2. Neuronal localization of AChE.

A to D: Cells expressing AChE. Wholemount cytochemical staining of AChE activity was performed on fixed sections of control (A, C) and transgenic (B, D) brains. Neuronal cell bodies in caudate-putamen (CP), globus pallidus (GP) (A, B) and hippocampal regions CA2 and CA3 (C, D) were stained more intensely in transgenic than in control brain. Size bars= 1mm (A,B) or 100 μm (C,D).

E, F: Dendritic accumulation of AChE. Cytochemical staining of AChE activity and electron microscopy were performed on fixed brain sections

including axo-dendritic terminals. Excess electron dense reaction products appeared as grains within dendrites (round structures) in transgenic brain (F) as compared with low density labeling in the control brain (E). Size bar= 0.5 μm.

Fig. 3. Transgenic mice display reduced hypothermic responses to cholinergic and serotonergic, but not adrenergic agonists.

Core body temperature was measured in 5 to 7 month old control and transgenic mice at the noted times after intraperitoneal injections of the marked doses of paraoxon (**A, B**), oxotremorine (**C**), nicotine (**D**), 8-OH-DPAT (**E**) or clonidine (**F**). Presented are average data for 5 (**A, F**) or 4 male and female mice (**C, D, E**) or one representative pair out of 3 tested (**B**). Note different time and temperature scales. Temperatures of transgenics at all time points after 10 min from injection were statistically different from those of controls for oxotremorine, nicotine and 8-OH-DPAT (student's t test, $p < 0.05$). Open circles: control mice, filled circles: transgenic mice.

Fig. 4. Progressive impairment in spatial learning and memory of HpACHE transgenic mice.

Mice were tested in a water maze with a hidden platform in a fixed location. Means of daily mean escape latencies are presented for 4 week old transgenic ($n = 10$) and control ($n = 5$) mice (**A**), 2 months old transgenic ($n = 10$) and control ($n = 10$) mice (**B**) and 6 to 7 months old transgenic ($n = 9$) and control ($n = 11$) mice (**C**). Stars note statistically significant reductions of latencies, as compared with the first day values in each section (one way ANOVA, followed by Neuman Keuls test, $p<0.02$).

Table I: Human AChE activities in tissues of control and transgenic mice

	AChE activity[1]			
	Transgenic		Control	
Tissue	Total	Bound to antibodies[2]	Total	Bound to antibodies[2]
Central nervous system				
basal forebrain	402 ± 25	201	187 ± 30	0
cortex	283 ± 18	119	235 ± 12	0
brainstem and cerebellum	95 ± 36	30	88 ± 25	0
spinal cord	255	82	208	0
Other				
adrenal gland	25	0	24.6	0
bone marrow	13.4 ± 0.3	0	17.1 ± 2.3	0

[1]Catalytic activities (nmol acetylthiocholine hydrolyzed/min/mg protein) were determined for detergent-solubilized homogenates as detailed [24]. Values are averages of 3 experiments with standard deviation, except for spinal cord and adrenal gland where single experiments are presented.

[2]Activity after binding to human AChE-specific monoclonal antibodies [24].

Table II: AChE overexpression does not modify length of axo-dendritic synapses in the anterior hypothalamus of transgenic mice.

Parameters	Controls	Transgenics	p (Student's t test)
Number of synapses (1)	32	12	-
Electron-dense deposits/μm^2 (2)	3.4 ± 3.2	6.5 ± 4.0	$p < 0.02$
Post-synaptic length in µm (3)	0.8 ± 0.3	0.7 ± 0.2	ns

(1) Electron microscopy and cytochemical staining of anterior hypothalamus tissue were as previously described [24] with modifications detailed under Materials and methods. Average values ± standard deviations are presented.

(2) Only dendritic electron-dense deposits with rigid limits, reflecting crystal structures, were counted.

(3) Post-synaptic length was measured only for those synapses associated with acetylthiocholine reaction products (i. e. cholinergic synapses). For a representative example, see Fig. 2C.

ns = non significant.

Table III: Selective behavioral deficits in AChE-transgenic mice

Behavioral parameter	Age, months					
	1		2		5-11	
	T	C	T	C	T	C
1. Water maze[1]						
a. visual test	33.8 ± 25.0	6.7 ± 1.8	22.5 ± 14.8	5.3 ± 2.2	33.3 ± 12.2	7.3 ± 3.9
b. hidden platform test	17.3 ± 12.5	8.7 ± 3.5	26.4 ± 21.3	14.0 ± 3.5	49.1 ± 27.8	20.5 ± 18.2
2. Explorative behavior[2]						
a. space explored	51.4 ± 19.7	40.3 ± 29.8	62.5 ± 33.6	43.7 ± 21.7	51.5 ± 17.2	54.1 ± 19.4
b. distance walked	11.2 ± 5.1	10.4 ± 8.5	15.9 ± 9.6	11.0 ± 5.4	13.0 ± 5.0	12.2 ± 3.5
3. Open field anxiety[3]						
a. Defecation	1.0 ± 0.6	0.6 ± 0.5	0.1 ± 0.4	0.5 ± 0.8	0.8 ± 0.4	0.4 ± 0.5
b. grooming	1.7 ± 1.0	0.6 ± 1.0	1.7 ± 1.0	1.0 ± 0.9	1.3 ± 1.0	1.2 ± 1.3

Notes to table III

(1) Noted are average escape latencies ± standard deviations at day 4 of a Morris water maze test.

(2) Explorative behavior in the open field test was evaluated by measuring the percentage of open field space explored out of 0.36 m2 and the distance covered in 5 minutes. Note no significant difference in both parameters between transgenics and controls.

(3) Open field tests were performed as described in methods. Defecation and grooming events, accepted signs of open field anxiety, were noted.

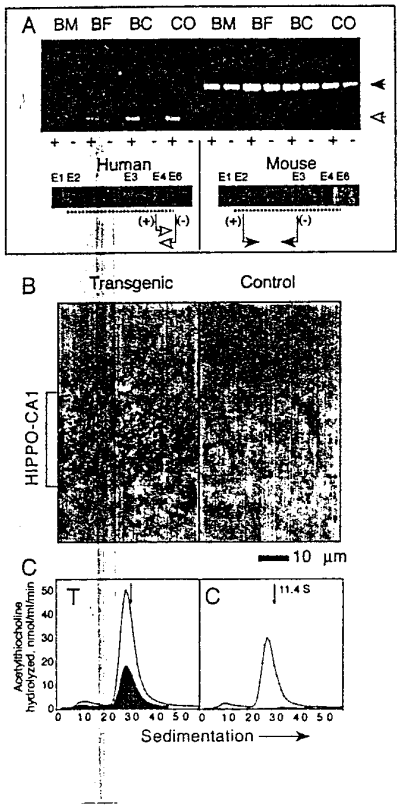

A
BM BF BC CO BM BF BC CO
Human
Mouse
E1 E2 E3 E4 E6
(+)
(-)
B
Transgenic
Control
HIPPO-CA1
10 μm
C
T
C
11.4 S
Acetylthiocholine hydrolyzed, nmol/ml/min
Sedimentation

Fig 1

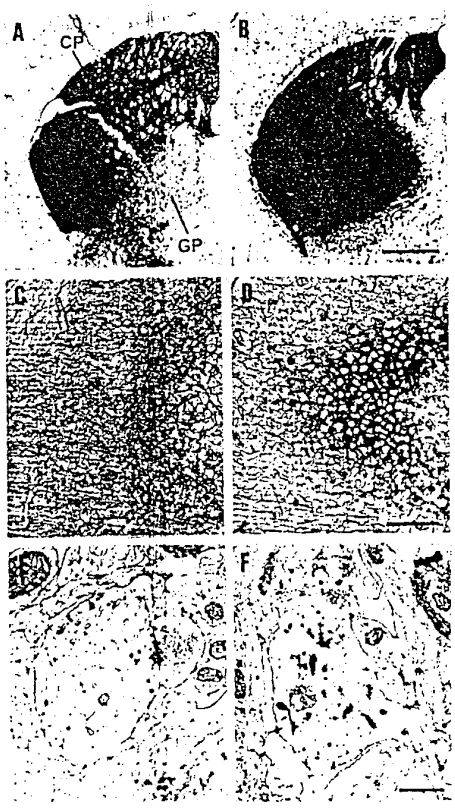

A
CP
GP
B
C
D
E
F

Fig 2

Fig 3

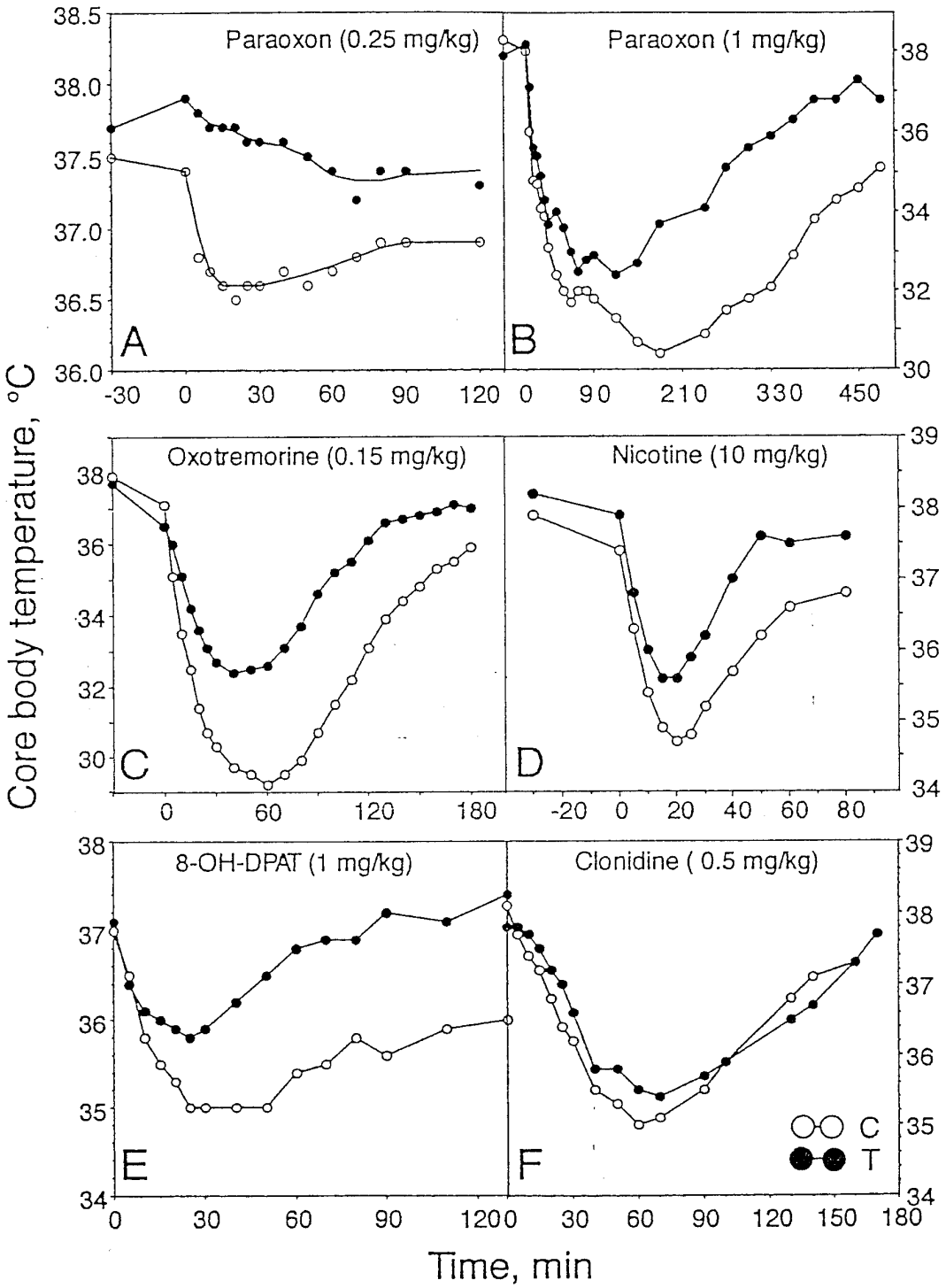
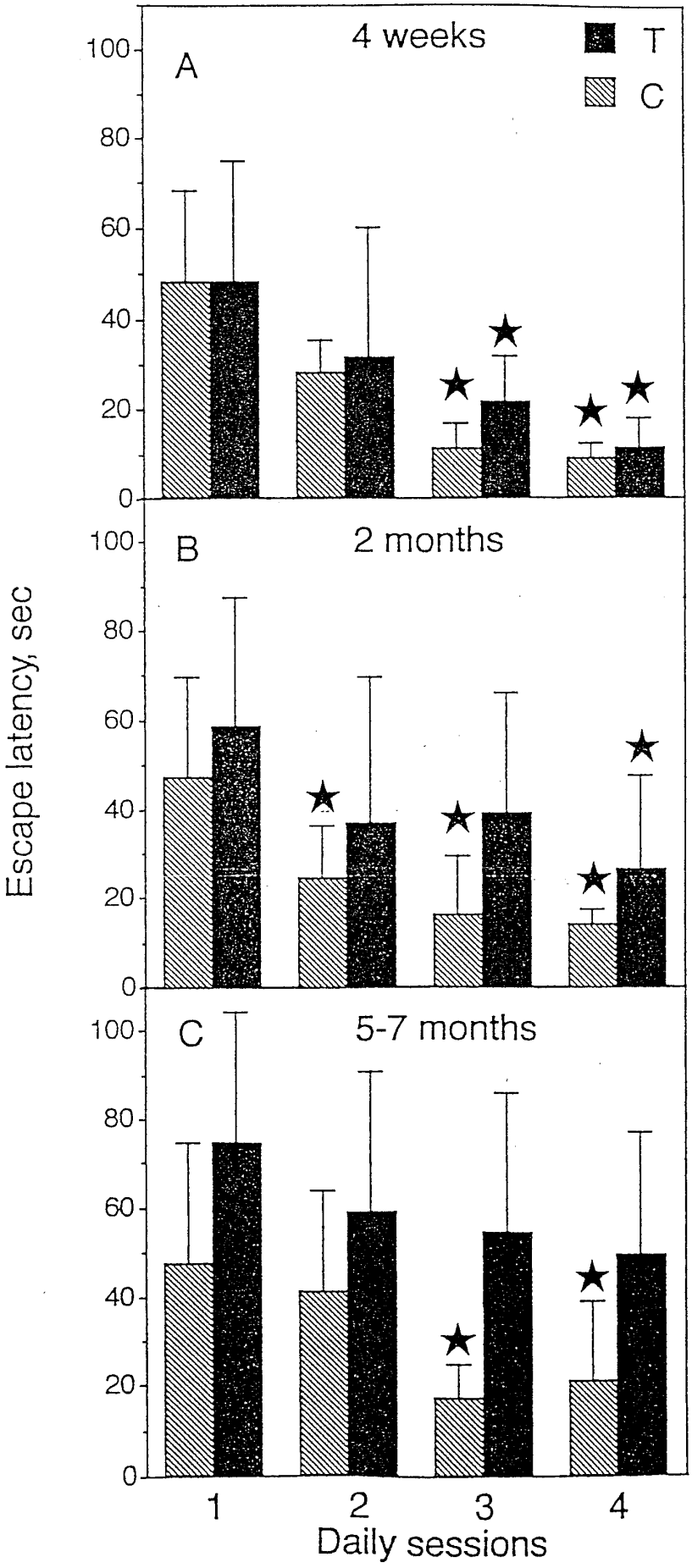

Paraoxon (0.25 mg/kg)
Paraoxon (1 mg/kg)
Oxotremorine (0.15 mg/kg)
Nicotine (10 mg/kg)
8-OH-DPAT (1 mg/kg)
Clonidine (0.5 mg/kg)
A
B
C
D
E
F
C
T
Core body temperature, °C
Time, min

Fig 4


4 weeks
T
C
A
B
2 months
C
5-7 months
Escape latency, sec
0
20
40
60
80
100
1
2
3
4
Daily sessions

GENETIC PREDISPOSITION TO ADVERSE CONSEQUENCES OF ANTI-CHOLINESTERASE THERAPIES ANTICIPATED IN CARRIERS OF THE "ATYPICAL" BUTYRYLCHOLINESTERASE MUTATION

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running head: Adverse anti-Cholinesterase Responses

ABSTRACT

Background: In search of the molecular basis of adverse responses to anti-cholinesterases (anti-ChEs), we have studied the family of an Israeli soldier who suffered various symptoms under prophylactic dosage of pyridostigmine during the Gulf War, following a previous incident of succinylcholine post-anesthesia apnea.DNA analyses identified this patient as being homozygous for the "atypical" allele of serum butyrylcholinesterase (BuChE), and his parents and sister as heterozygous carriers. This raised the possibility that his adverse symptoms were related to his BCHE genotype.
Methods: Serum BuChEs from the soldier, his father and individuals homozygous for normal BuChE were subjected to inhibition by several anti-ChEs, either in solution or immobilized via antibodies. Recombinant normal and "atypical" human BuChE and acetylcholinesterase (AChE), were tested separately or in mixtures mimicking the heterozygous state.
Results: Both native and recombinant "atypical" BuChEs were significantly less sensitive than normal BuChE to several anti-ChEs: tetrahydroaminoacridine (THA, tacrine, Cognex®), the reversible AChE inhibitor recently approved for treatment of Alzheimer's disease (by two orders of magnitude) and the carbamates, pyridostigmine, physostigmine, heptyl physostigmine, and SDZ-ENA 713 (by 2.5-, 15-, 14-, and 30-fold, respectively). Moreover, tacrine, SDZ-ENA 713, and heptyl physostigmine all reacted with AChE with lower affinity and/or far slower than with both native and recombinant normal, yet not with "atypical" BuChE.
Conclusions: These findings imply that AChE is a particularly vulnerable target for anti-ChEs in individuals homozygous for the "atypical" BCHE allele (up to 0.6% of some populations). This may explain the effect of the drug on some of the soldiers treated with pyridostigmine and on some of the Alzheimer's patients treated with tacrine who responded adversely, and suggest particular serum and DNA tests to identify individuals at risk for such responses.

key words: acetylcholinesterase, Alzheimer's disease, anti-cholinesterase, "atypical" butyrylcholinesterase, pyridostigmine, tacrine

INTRODUCTION

The clinical uses of anti-cholinesterases (anti-ChEs) have recently been extended in two circumstances, involving large numbers of subjects. First, during the 1991 Gulf War the carbamate, pyridostigmine was administered prophylactically to over 400,000 soldiers in anticipation of nerve agent attacks, with the intention of transiently blocking, and thus protecting, a fraction of their nervous system acetylcholinesterase (AChE, EC 3.1.1.7)(1, 2). More recently, the reversible cholinesterase (ChE) inhibitor, tetrahydroaminoacridine (THA, tacrine, Cognex®) was approved for use in patients with Alzheimer's disease, who suffer from massive degeneration of cholinergic neurons (3), for the purpose of enhancing the availability of acetylcholine at synapses and improving residual cholinergic neurotransmission in these patients. Adverse symptoms were reported in a subset of individuals in both groups (4-7), including responses associated with the cholinergic syndrome, such as nausea, and also depression, general fatigue, insomnia, and weight loss. However, these are only a few of many symptoms in a complex and diverse list. Interpretation of these symptoms is complicated by incomplete medical records (4) and the stressful situation experienced by the first group and the generally bad condition of the aging patients from the second group. To identify the molecular basis of these adverse responses to anti-ChEs in of the treated individuals, we have focused on the enzyme targets of these agents.

Anti-ChEs have been designed primarily as selective AChE inhibitors. However, many of these drugs also interact quite efficiently with the closely related serum enzyme butyrylcholinesterase (BuChE, EC 3.1.1.8). In fact, some consider one of BuChE's biological roles to be a scavenger of natural anti-ChEs (for a recent review see ref. 8). This scavenging capacity, in turn, may depend on BuChE's genetic variation. There are over 20 known allelic variants of BCHE (the gene encoding BuChE), some enzyme products of which display altered interactions with certain inhibitors (9-11). In contrast, no allelic variant with modified biochemical properties is known for AChE, perhaps because the fully active enzyme is absolutely essential to cholinergic neurotransmission. This raised the possibility that reduced scavenging capacities led to the adverse symptoms experienced by some patients undergoing anti-ChE therapies, and that these capacities are rooted in the genetic polymorphism of BuChE.

The most common allele of BCHE that has a variant phenotype is the substitution of aspartate at position 70 by glycine. This allele is known as the "atypical" BCHE variant (9, 12, 13). The average allele frequency of this variant is under 2% in Europe (14) but up to 7.5% in some sub-populations originating in the Middle East (15), which indicates that 1:2,500 and 1:180, respectively, among these populations will be homozygous for the mutation. The variant is much less frequent in other populations, such as sub-Saharan Africans (14).

The "atypical" variant, in contrast to the normal enzyme, is unable to hydrolyze succinylcholine, and is much less sensitive to several inhibitors: physostigmine (16) and several organophosphates, including diisopropylfluorophosphonate (DFP), tetraisopropyl pyrophosphoramide (iso-OMPA), and diethoxyphosphinylthiocholine (echothiophate) (8, 10, 17). Homozygous carriers of this variant allele were reported to be particularly sensitive to succinylcholine at surgery, which causes in them post-anesthesia apnea (18). They also display hypersensitivity to the insecticide parathion (reviewed in ref. 19). Individuals carrying this allele would hence be logical candidates for adverse effects of anti-ChE therapies. This issue arose when we learned of an individual who had experienced both succinylcholine-induced apnea and, during the Gulf War under treatment with pyridostigmine, cholinergic symptoms. The succinylcholine incident indicated the variant allele, and we suspected that the cholinergic symptoms reflected the patient's response to anti-ChE therapy and might have the same genetic basis. Therefore, we initiated a study of the interaction of ChE inhibitors in clinical use or testing, with serum ChEs from members of this subject's family, and compared these results with studies using the enzyme from non-variant serum and with recombinantly produced variant ChEs.

METHODS

All procedures followed were in accord with the Helsinki Declaration as revised in 1983 and with local Institutional guidelines for the care and use of laboratory animals.

Patients and methods: A.B. (name and details in hospital Medical Records), born in 1970, was first referred to us in 1989 following an incident of post-anesthesia apnea. Anesthesia was induced by Na pentothal (250 mg) and succinylcholine (75 mg). During anesthesia benzodiazepam (5 mg), petidine (50 mg), beatryl (0.1 mg) and atropine (0.5 mg) were administered. After 30 min of surgery (closed reduction of bilateral tibial fractures), mechanical respiration was continued for 5 hr due to failure to breathe spontaneously. During this period the electrocardiogram and pulse remained normal, mean blood pressure was 130:80 and blood glucose, urea, Na and K values were normal. Hemoglobin count was 12 g/dl, with slight leukocytosis prior to surgery (14,000 cells/dl), yet with normal temperature. One day after surgery, the BuChE dibucaine number (16) was determined to be 23% of normal. A.B. was released following 9 days of uneventful hospitalization. We then found his serum BuChE to have approximately 30% of normal capacity for butyrylthiocholine (BTCh) hydrolysis and no capacity for binding succinylcholine, suggesting that it was the "atypical" enzyme (9, 12,13). Following PCR amplification, *SauIII*A restriction and direct sequencing of the corresponding region from the BCHE gene isolated from his venous blood DNA by established techniques (15), A.B. was diagnosed as being homozygous for the "atypical" allele, but not a carrier of other frequent point mutations of BuChE. The same methods revealed that both his parents and his sister were heterozygous carriers of the "atypical" BCHE allele. The patient was advised to avoid anti-ChE drugs or insecticides. A.B. served in the Israel Defense Forces in 1991, during the period of the Gulf War and, with others, thrice daily received 30 mg prophylactic doses of pyridostigmine. He developed nausea, insomnia, weight loss, and general fatigue, which worsened consistently, and a deep depression. Following discontinuation of pyridostigmine, his condition improved gradually over the following weeks and A.B. is currently without symptoms.

Variant enzymes: Serum BuChE activity against BTCh was measured spectrophotometrically as detailed previously (10, 13). Recombinant normal and "atypical" BuChEs were produced in *Xenopus* oocytes microinjected with *in vitro*-transcribed BCHEmRNAs prepared from the corresponding cDNA types (10). Alternative recombinant AChEs were produced in ACHEDNA-injected oocytes under control of the cytomegalovirus (CMV) promoter, using either the brain-characteristic 3'-exon 6 (20) or the blood cell-expressed domain composed of the fourth pseudo-intron and the 3'-exon 5 (21).

Inhibitors: Tacrine and physostigmine were purchased from Sigma Chemical Co. (St. Louis, MO). SDZ-ENA 713 and heptyl physostigmine were gifts of Sandoz (Bern, Switzerland) and Merck Sharp & Dohme (Harlow, U.K.), respectively. Pyridostigmine bromide was from Research Biochemicals International (Natik, MA).

Antibody immobilizations: Monoclonal mouse anti-human serum BuChE (no. 53-4) or anti-human AChE (no. 101-1), 4 μg/ml, were adsorbed to multiwell microtiter plates overnight at 4 °C in carbonate buffer (22). Free binding sites were blocked with PBS-T buffer (144 mM NaCl, 20 mM Na phosphate, pH 7.4, 0.05% Tween 20, and 0.01% thimerosal) for 60 to 80 min at 37 °C. Homogenates of microinjected oocytes or serum samples were diluted 1:20 to 1:40 in PBS-T to achieve similar activity levels and were incubated in the antibody-coated wells for 4 hr at room temperature with agitation, and overnight at 4 °C. Plates were washed 3 times with PBS-T prior to use.

Inactivation and reactivation measurements: IC_{50} values were determined essentially as described (23), assaying soluble enzyme in the presence of tacrine against 1 mM acetylthiocholine (ATCh). For inactivation and reactivation by carbamates, antibody-immobilized enzymes were exposed to the tested anti-ChE in PBS-T buffer for varying times (0.5 to 80 min) at room temperature following an initial determination of catalytic activities. At the noted times, plates were washed 3 times with PBS-T and remaining substrate hydrolysis rates were determined (23). Spontaneous reactivation at room temperature was

measured for immobilized recombinant ChEs following complete inhibition, 3 washes with PBS-T, subsequent incubation and activity determination at the noted times.
Specific activity: To determine enzyme quantities, BuChE immobilized *via* mouse anti-human serum BuChE (no. 53-4) was incubated with a rabbit anti-human polyclonal antiserum (Dako, Glostrup, Denmark) at 1:4,000 dilution in PBS-T for 70 to 80 min at 37 °C. After washing with PBS-T, horseradish peroxidase-conjugated goat anti-rabbit antibody (Jackson Laboratory, Bar Harbor, ME) was added at 1:10,000 dilution in PBS-T. Peroxidase activity was thereafter assayed using *o*-phenylenediamine dihydrochloride at 1 mg/ml in 0.05 M phosphate/citrate buffer, pH 5.0, with 0.03% Na perborate. Purified human BuChE was used for calibration and change of absorbance at 450 nm was recorded on a Molecular Devices (Menlo Park, CA) microtiter plate reader.

RESULTS

We first examined BuChE from sera of A.B. and his father, previously identified as homozygous and heterozygous carriers of the "atypical" BCHE allele, respectively. We then compared these enzymes to BuChEs from individuals homozygous for the normal BCHE allele. To analyze on a micro scale the interactions of covalently interacting inhibitors with ChEs, we immobilized native human BuChEs through monoclonal antibodies to multiwell microtiter plates. For reference, we also immobilized recombinant *Xenopus* oocyte-produced variant ChEs, including normal and "atypical" BuChEs (13) and brain- and blood cell-characteristic AChEs (21). The enzymes, immobilized in multiwell plates, were subjected to inactivation by an anti-ChE. By this procedure inhibition rates could be conveniently determined, unbound inhibitors could be removed prior to activity measurements, and amounts of the enzyme could be determined for each sample. In some experiments enzymes were allowed to spontaneously reactivate following inhibition.

Normal enzyme hydrolyzed 81 ± 23 nmol BTCh/hr/µl serum (assayed at 2 mM substrate, an average from 20 individuals), while the activity of "atypical" BuChE was found to be about 3-fold lower in A.B.'s serum, which contained a normal amount of enzyme protein. This is in agreement with values obtained previously for other patients homozygous for the "atypical" allele (18). Recombinant "atypical" and normal BuChEs also confirmed this difference in specific activities (13). Heterozygotes presented intermediate specific activities, 60-70% of normal homozygotes (average of 3 genetically confirmed individuals). Thus, carriers of the "atypical" allele have a less active BuChE, although they carry amounts of serum BuChE protein (approx. 20 ng/µl) similar to individuals with the normal enzyme.

To test the interactions of "atypical" BuChE with various anti-ChEs, in 1994 we prepared fresh serum samples from A.B.'s peripheral blood and from his heterozygous father. Serum from genetically diagnosed normal homozygotes served for comparison. Inactivation measurements demonstrated that "atypical" BuChE reacts much slower than does its normal counterpart with four carbamates, pyridostigmine (1), physostigmine (24), heptyl physostigmine (25) and SDZ-ENA 713 (26) (Fig. 1A). Moreover, differences between the serum enzyme of the heterozygous father and that of homozygous normal sera were also evident in the inactivation rates. Both these effects varied with the particular inhibitor being tested. For example, "atypical" BuChE displayed a decrease of approx. 40% in its activity when incubated with SDZ-ENA 713 for 30 min, as compared with an 95% loss of activity in the normal enzyme at this time, and with 80% in the enzyme from heterozygous serum (Fig. 1A). The loss and rate of loss of BuChE activity, thus, depend significantly on the individual's genotype. To evaluate the actual scavenging capacity of each enzyme type for the various drugs, we took into account the reduced specific activity of the "atypical" protein and calculated amounts of inactivated enzyme per volume serum as a function of time. These calculations reveal drastically different capacities for scavenging each of the tested drugs in sera of individuals with the normal and the "atypical" alleles, as illustrated in Fig. 1B.

To examine whether such genetic predisposition to altered drug interactions can also be expected for the reversible Alzheimer's disease drug, tacrine (27), we determined the IC_{50} values for this drug (Fig. 2A and Table 1). To test whether the observed differences were

indeed due to the examined point mutation, similar dose-dependence curves were also prepared for recombinant, *Xenopus* oocyte-produced normal and "atypical" monomeric BuChE. To mimic the heterozygous state, 1:1 mixtures of the normal and the "atypical" enzyme were tested (equal volumes of oocyte homogenates, which was close to equal amounts of the ChEs) (Fig. 2B). In both cases, we observed a drastic reduction in the capacity of "atypical" BuChE, as compared with the normal enzyme, to interact with tacrine. Mixtures of normal and "atypical" enzyme, whether from the heterozygous sera or prepared by genetic engineering, yielded the expected intermediary inhibition curves (Fig. 2B). As the oocyte-produced enzyme remains primarily monomeric (19), these differences were apparently not affected by multisubunit assembly. Also, competition between the two types of enzyme subunits, which might differ between the native and recombinant states, was apparently ineffective with respect to tacrine interactions. Thus, for tacrine as well, one might expect drastically different scavenging capacities of serum BuChE, depending upon the genotype of the individual. For example, in the presence of 1 μM tacrine, normal BuChE is completely inhibited whereas the "atypical" enzyme shows virtually no inhibition, and sera from heterozygotes show intermediate levels of activity.

To search for the molecular basis of the differing interactions of tacrine with AChE and the normal and the "atypical" BuChEs, we referred to the 3-dimensional structures. Within the computer-modeled structure of human BuChE (28), Asp70 emerges close to the rim of the active site gorge (Fig. 3, top), where it can contact approaching ligands prior to their entrance to the active site itself. Expansion of the relevant amino acid residues in human BuChE (Fig. 3, bottom left), as compared with the corresponding crystal region in tacrine-soaked *Torpedo* AChE (ref. 29; Fig. 3, bottom right), reveals that Phe330, one of the AChE residues quite close to tacrine is substituted by Ala328 in BuChE. This is reflected in tacrine's slightly smaller IC_{50}, 0.05 mM for BuChE *vs.* 0.15 mM for AChE in normal BuChE. Also, the distance from the Asp70 carboxyl group to the tacrine anilinic nitrogen is only 3.7 Å, indicating the possibility of a salt bridge. This interaction is removed in "atypical" BuChE, reflected in a two orders of magnitude increase in tacrine's IC_{50} (Table 1).

In addition to inactivation and removal rates and to general hemodynamic parameters, the dynamic level of drug concentrations in a patient's serum depends on the rate of reactivation of drug-enzyme complexes. To get a more complete picture of the expected outcome of treating genetically-distinct individuals with anti-ChEs, we also used the antibody-immobilized recombinant enzymes to follow spontaneous rates of reactivation for each of the examined drugs (Fig. 4). These measurements revealed considerable differences among drugs and between AChE and BuChE reactivation rates for each drug. However, for no drug was there a dramatic difference between the blood and brain forms of AChE or between normal and "atypical" BuChE (Fig. 4). Of the examined drugs, pyridostigmine reactivation reached 40 and 10% for AChE and BuChE, respectively, within 50 min. These reactivation rates for AChE were considerably lower than those determined for physostigmine, an effect reflected in the short *in vivo* half life of this drug (30, 31). While physostigmine-inhibited AChE reactivated up to 90% in 45 min, in that time normal and "atypical" BuChE regained only 10 or 20% of their original activities. Reactivation of both heptyl physostigmine-inhibited BuChE forms was et slower, and it could reactivate by only 10%, while AChE reactivation wasas low as 5%. Finally, SDZ-ENA 713 reactivation levels for all of the examined enzymes never exceeded 8% within one hour (Fig. 4). In view of the correlation with published *in vivo* half-lives (32), we conclude that combined inactivation (Table 2) and reactivation (Fig. 4) rates determined *in vitro* for the recombinant and native enzymes, may together predict the fate of these drugs in patients' sera *in vivo*.

DISCUSSION

The current family study and recombinant enzyme analyses predict that anti-ChE therapies may cause adverse reactions in some individuals, depending on their BCHE genotype. This possibility becomes an urgent issue in view of the recent large scale use of pyridostigmine in soldiers on the one hand (1, 2, 33), and the use of tacrine for Alzheimer's disease patients on the other (3, 6, 34).

Our study was conducted with antibody-immobilized ChEs, which enabled us to stop the inactivation or reactivation processes at any given time, remove excess inhibitor, and measure ChE activities for obtaining correct rates of these processes. While this approach does not take into consideration pharmacodynamics, it does provide accurate values for the target molecule, *i.e.* the human ChEs themselves. In previous studies, inhibition levels but not rates of inhibition were determined, and activities were assessed in the presence of the inhibitors, which change them. A second major feature distinguishing our study from previous ones is the comparison with human recombinant ChEs. Inhibition observed for immobilized serum enzymes, as confirmed with the recombinant enzyme, may with confidence be taken as a true interaction of inhibitor and enzyme.

The recent administration of pyridostigmine bromide to over 400,000 Gulf War soldiers was probably the largest scale ever use of an experimental drug (35), approved because of the anticipated exposure of those soldiers to nerve agents (2). Previous clinical studies with this drug on healthy volunteers, who showed no adverse effects, had been limited to small numbers of healthy males and to only several days exposure *(e.g.* ref. 33). Thus the Gulf War presents the first real-life experience with this anti-ChE. The war conditions included concurrent exposure to insecticides, chiefly organophosphorus anti-ChEs (4), which further increased the level of ChE inhibition in these soldiers to an unknown extent. It is thus possible that the total ChE inhibition levels in these soldiers sometimes exceeded those planned even for normal individuals. In homozygotes and possibly in heterozygous carriers of the "atypical" BCHE allele, the capacity of blood BuChE to interact with and detoxify some of the drug would be significantly lower (Fig. 1B), resulting in larger effective doses. While heterozygotes still possess about 50% of this normal protective detoxifier, "atypical" homozygotes have practically none. These would hence become most vulnerable to ChE inhibition under treatment by any anti-ChE drug.

Pyridostigmine is not the only anti-ChE that may provoke an adverse response, as is demonstrated by our analyses of several Alzheimer's disease drugs. All of these drugs emerged as much faster inactivators of BuChE than of AChE, suggesting that when orally administered to patients, these drugs will interact primarily with plasma BuChE. The clinically effective dose reaching the central nervous system depends heavily, therefore, on the BCHE genotype. Even without the complication of BCHE polymorphism, BuChE levels can vary with the general state of health (14). Since Alzheimer's patients are far from being as healthy as the pyridostigmine-treated soldiers, they may present yet more drastic symptoms in response to inappropriate dosage of anti-ChEs. Moreover, these patients are usually unable to communicate their difficulties, so that discrimination between effects of the drug and symptoms of the disease depends solely on the subjective observations of a caretaker.

Our analyses predict different effects of the "atypical" genotype on individual responses to several Alzheimer's drugs. With all of the tested carbamates, significant differences are to be expected in homozygous "atypicals", and less so in heterozygotes, due to the over 10-fold differences in the inactivation rates by these drugs of normal and "atypical" BuChE. In addition to the 0.04 to 0.6% homozygotes among the treated populations, difficulties may also be predicted for those heterozygotes suffering from liver malfunction and reduced BuChE levels from other causes. The situation would be exacerbated under treatment with tacrine. This drug reacts with "atypical" BuChE so much more weakly than with its normal counterpart that the "atypical" enzyme becomes a negligible factor in attenuating its interaction with AChE. Under these circumstances even heterozygotes might show adverse responses, as their AChE levels would be reduced because of lack of scavenger under doses that cause no reduction in homozygous normals. The reported high percentage of cholinergic symptoms under tacrine treatment (up to 15%) may perhaps reflect such heterozygotes and, in addition, patients with liver malfunction and consequently with low serum BuChE levels.

In addition to short-term effects, long-term adverse consequences of treatment with either type of anti-ChE may be associated with the "atypical" BCHE genotype. These possibilities

can be easily tested. Combined genotype/phenotype tests for "atypical" BCHE/BuChE are available (9, 15, 18) and are relatively simple to perform. If indeed the genotype of patients determines their response to anti-ChEs, as we predict based on the above experiments, the incidence of the "atypical" BCHE allele should be significantly higher among Alzheimer's patients with adverse short- and/or long-term reactions to tacrine and among veterans who experienced adverse effects to pyridostigmine. Should this be the case, appropriate dosages of these drugs might be adjusted to match specific genotypes, for the purpose of avoiding these adverse responses.

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REFERENCES

1. Keeler JR, Hurst CG, Dunn MA. Pyridostigmine used as a nerve agent pretreatment under wartime conditions. J Am Med Assn 1991;266:693-5.
2. Gavageran, H. NIH panel rejects Persian Gulf Syndrome, Nature 1994;369:8.
3. Knapp MJ, Knopman DS, Solomon PR, Pendlebury WW, Davis CS, Gracon SI. A 30-week randomized controlled trial of high-dose Tacrine in patients with Alzheimer's disease. J Am Med Assn 1994;271:985-91.
4. Cotton P. Veterans seeking answers to syndrome suspect they were goats in Gulf War. J Am Med Assn 1994;271:1559-61.
5. Sharabi Y, Danon Y, Berkenstadt H et al. Survey of symptoms following intake of pyridostigmine during the Persian Gulf War. Isr J Med Sci 1991;27:656-8.
6. Beermann B. Side effects of long acting cholinesterase inhibitors. Acta Neurol Scand 1993;Suppl. 149,88:53-4.
7. Winker MA. Tacrine for Alzheimer's disease; which patient, what dose? J Am Med Assn 1994;271:1023-4.
8. Schwarz M, Glick D, Loewenstein Y, Soreq H. Engineering of human cholinesterases explains and predicts diverse consequences of administration of various drugs and poisons. Pharmacol. Therap. (in press).
9. McGuire MC, Nogueira CP, Bartels C et al.. Identification of the structural mutation responsible for the dibucaine-resistant (atypical) variant form of human serum cholinesterase. Proc Natl Acad Sci U S A 1989;86:953-7.
10. Neville LF, Gnatt A, Padan R, Seidman S, Soreq H. Anionic site interactions in human butyrylcholinesterase disrupted by two adjacent single point mutations. J Biol Chem 1990;265:20735-8.
11. Masson P, Adkins S, Gouet P, Lockridge O. Recombinant human butyrylcholinesterase G390V, the fluoride-2 variant, expressed in Chinese hamster ovary cells, is a low affinity variant. J Biol Chem 1993;268:14329-41.
12. Neville LF, Gnatt A, Loewenstein Y, Soreq H. Aspartate-70 to glycine substitution confers resistance to naturally occurring and synthetic anionic-site ligands on in-ovo produced human butytrylcholinesterase. J Neurosci Res. 1990;27:452-60.
13. Neville LF, Gnatt A, Loewenstein Y, Seidman S, Ehrlich G, Soreq H. Intramolecular relationships in cholinesterase revealed by oocyte expression of site-directed and natural variants of human BCHE. EMBO J 1992;11:1641-9.
14. Whittaker, M. Cholinesterase. Basel: Karger, 1986.
15. Ehrlich G, Ginzberg D, Loewenstein Y et al. Population diversity and distinct haplotype frequencies associated with ACHE and BCHE genes of Israeli Jews from transcaucasian Georgia and from Europe. Genomics 1994;22:288-95.
16. Kalow W, Davis RO. The activity of various esterase inhibitors towards atypical human serum cholinesterase. Biochem Pharmacol 1958;1:183-92.
17. La Du BN, Bartels CF, Nogueira C et al. Phenotypic and molecular biological analysis of human butyrylcholinesterase variants. Clin Biochem 1990;23:423-31.

18. Lockridge O. Genetic variants of serum cholinesterase influence metabolism of the muscle relaxant succinylcholine. Pharmacol Ther 1990;47:35-60.
19. Soreq H, Zakut H. Human Cholinesterases and Anticholinesterases. San Diego: Academic Press, 1993.
20. Soreq H, Ben-Aziz R, Prody CA et al. Molecular cloning and construction of the coding region for human acetylcholinesterase reveals a G+C-rich attenuating structure. Proc Natl Acad Sci U S A 1990;87:9688-92.
21. Karpel R, Ben Aziz-Aloya R, Sternfeld M et al. Expression of three acetylcholinesterase messenger RNAs in human tumor cell lines of different tissue origins. Exp Cell Res 1994;210:268-77.
22. Seidman S, Ben Aziz-Aloya R, Timberg R et al. Overexpressed monomeric human acetylcholinesterase induces subtle ultrastructural modifications in developing neuromuscular junctions of *Xenopus laevis* embryos. J Neurochem 1994;62:1670-81.
23. Loewenstein Y, Gnatt A, Neville LF, Soreq H. A chimeric human cholinesterase: identification of interaction sites responsible for sensitivity to acetyl- or butyrylcholinesterase-specific ligands. J Mol Biol 1993;234:289-96.
24. Thomsen T, Zendeh B, Fischer JP, Kewitz H. In vitro effects of various cholinesterase inhibitors on acetyl- and butyrylcholinesterase of healthy volunteers. Biochem Pharmacol 1991;41:139-41.
25. Iversen LL. Approaches to cholinergic therapy in Alzheimer's disease. Prog Brain Res 1993;98:423-6.
26. Enz A, Amstutz R, Boddeke H, Gmelin G, Malonowski J. Brain selective inhibition of acetylcholinesterase: a novel approach to therapy for Alzheimer's disease. Prog Brain Res 1993;98:431-7
27. Gregor VE, Emmerling MR, Lee C, Moore CJ. The synthesis and *in vitro* acetylcholinesterase and butyrylcholinesterase inhibitory activity of Tacrine (Cognex®) derivatives. Bioorgan Med Chem Lett 1992;2:861-4.
28. Harel M, Sussman JL, Krejci E et al. Conversion of acetylcholinesterase to butyrylcholinesterase: modeling and mutagenesis. Proc Natl Acad Sci U S A. 1992;89:10827-31.
29. Harel M, Schalk I, Ehret-Sabatier Let al. Quaternary ligand binding to aromatic residues in the active-site gorge of acetylcholinesterase. Proc Natl Acad Sci U S A 1993;90:9031-5.
30. Giacobini E. The second generation of cholinesterase inhibitors: pharmacological aspects. In: Cholinergic Basis for Alzheimer Therapy, Becker R and Giacobini E, eds., Birkhauser, Boston, 1991, pp. 247-62.
31. Johansson IM, Nordberg. Pharmacokinetic studies of cholinesterase inhibitors. Acta Neurol Scand 1993; 88,Suppl. 149:22-5.
32. Hartvig P, Wiklund L, Aquilonius SM, Lindstrom B. Clinical pharmacokinetics of centrally acting cholinesterase inhibitors. In: Cholinergic Basis for Alzheimer Therapy,. Becker R and Giacobini E, eds., Birkhauser, Boston, 1991, pp. 68-73.
33. Glikson M, Achiron A, Ram Z, Ayalon A, Karni A. The influence of pyridostigmine administration on human neuromuscular function. Fund Appl Toxicol 1991:16:288-98.
34. Watkins PB, Zimmerman HJ, Knapp MJ, Gracon SI, Lewis KW. Hepatotoxic effects of Tacrine administration in patients with Alzheimer's disease. J Am Med,Assn 1994;271:992-8.
35. Caldwell JA Jr. A brief survey of chemical defense, crew rest, and heat stress/physical training issues related to Operation Desert Storm. Mil Med 1992;157:275-81.

LEGENDS FOR FIGURES

Fig. 1. Inhibition of BuChE in human sera by carbamate anti-ChEs. A. Percent original activity as a function of time of exposure to 4 carbamates. In a representative experiment, the activities of immobilized ChEs were determined in 10 mM BTCh following incubation for the noted times with the covalently binding inhibitors pyridostigmine (10 μM), physostigmine (1 μM), heptyl physostigmine (0.01 μM), and SDZ-ENA 713 (10 μM). The lines are best fits of the data to first order conditions. **B.** Calculated scavenging capacity consumed by each enzyme for the 4 carbamate inhibitors. Calculations were based on the data of Fig. 1A and the average specific activity of the different enzymes. Inactivated enzyme is expressed as units of activity per volume lost during incubation. The concentration of enzyme protein was approximately the same in all serum samples. ▲, normal, Asp70, BuChE homozygote (D/D); ■ "atypical", Gly70, homozygote (G/G); ●, BuChE heterozygote (G/D).

Fig. 2. Inhibition of serum and recombinant BuChE by tacrine. In the left panel, data for 3 representative serum types inhibited by tacrine: serum of the propositus, A.B., a homozygote for the "atypical", Gly70, BuChE variant (G/G); of a heterozygote for this variant (G/D); and of a normal homozygote with Asp70 (D/D). In the right panel, tacrine inhibition is observed on equivalent total amounts of recombinant normal (D/D) and "atypical" (G/G) BuChE, and a 1:1 mixture of the two (D/G).

Fig. 3. ChE structures and tacrine binding . Upper: Three-dimensional ribbon structure of BuChE. The figure shows several residues as space-filling models. The active site gorge lies in the plane of the figure, with its opening at the top. **Lower:** Residues surrounding tacrine (THA). The 3-dimensional structure of *Torpedo* AChE crystals soaked with tacrine (right), is compared to the homologous residues of BuChE (left). The representation of human BuChE without and with tacrine was drawn after Harel *et al.* (28,29) using Insight II (Biosym Technologies).

Fig. 4. Time-dependent reactivation of recombinant ChEs. Spontaneous reactivation of recombinant human ChEs was examined after complete inhibition of the immobilized enzymes, followed by removal of unreacted inhibitor. Values of percent regained activity are shown. The lines are best fits to first order conditions (average of two experiments).

TABLE 1. Affinity of tacrine for cholinesterases; IC_{50} values (μM)[a]

		recombinant	serum
BuChE	normal	0.054 ± 0.036	0.082 ± 0.009
	"atypical"	11.4 ± 1.4	8.9 ± 2.5
AChE	brain-type (E6)	0.15 ± 0.08	---
	blood cell-type (E5)	0.15 ± 0.04	---

[a]IC_{50} values of tacrine were measured for recombinant human ChEs and for sera in the presence of 1 mM BTCh for BuChE or 1 mM ATCh for AChE. Values are calculated by GraFit 3.0 (Erithacus Software, Staines, UK). The data shown are averages of 2 serum samples or 3 recombinant enzyme samples ± standard deviation.

TABLE 2. Rates of reaction of cholinesterases with carbamate inhibitors; second order rate constants ($M^{-1} min^{-1}$)[a].

	BuChE		AChE	
	normal	"atypical"	E6	E5
pyridostigmine (10^{-5} M)	1.9 ± 0.2	<0.8	22 ± 9	25 ± 7
physostigmine (10^{-6} M)	380 ± 160	26 ± 9	ND	ND
heptyl physostigmine (10^{-8} M)	11,000 ± 3,000	770 ± 440	1,600 ± 700	1,400 ± 400
SDZ-ENA 713 (10^{-5} M)	14 ± 3	0.47 ± 0.46	4.3 ± 1.8	3.3 ± 0.6

[a]Pseudo first-order rate constants were extracted for the recombinant enzymes from data such as those in Fig. 1 with the reagent concentrations in parentheses. Data were fit to a first order decay model using the least squares approach. Averages and standard deviations of at least 4 determinations are presented. ND, not determined because of the exceedingly fast reactivation rates.

Fig. 1:

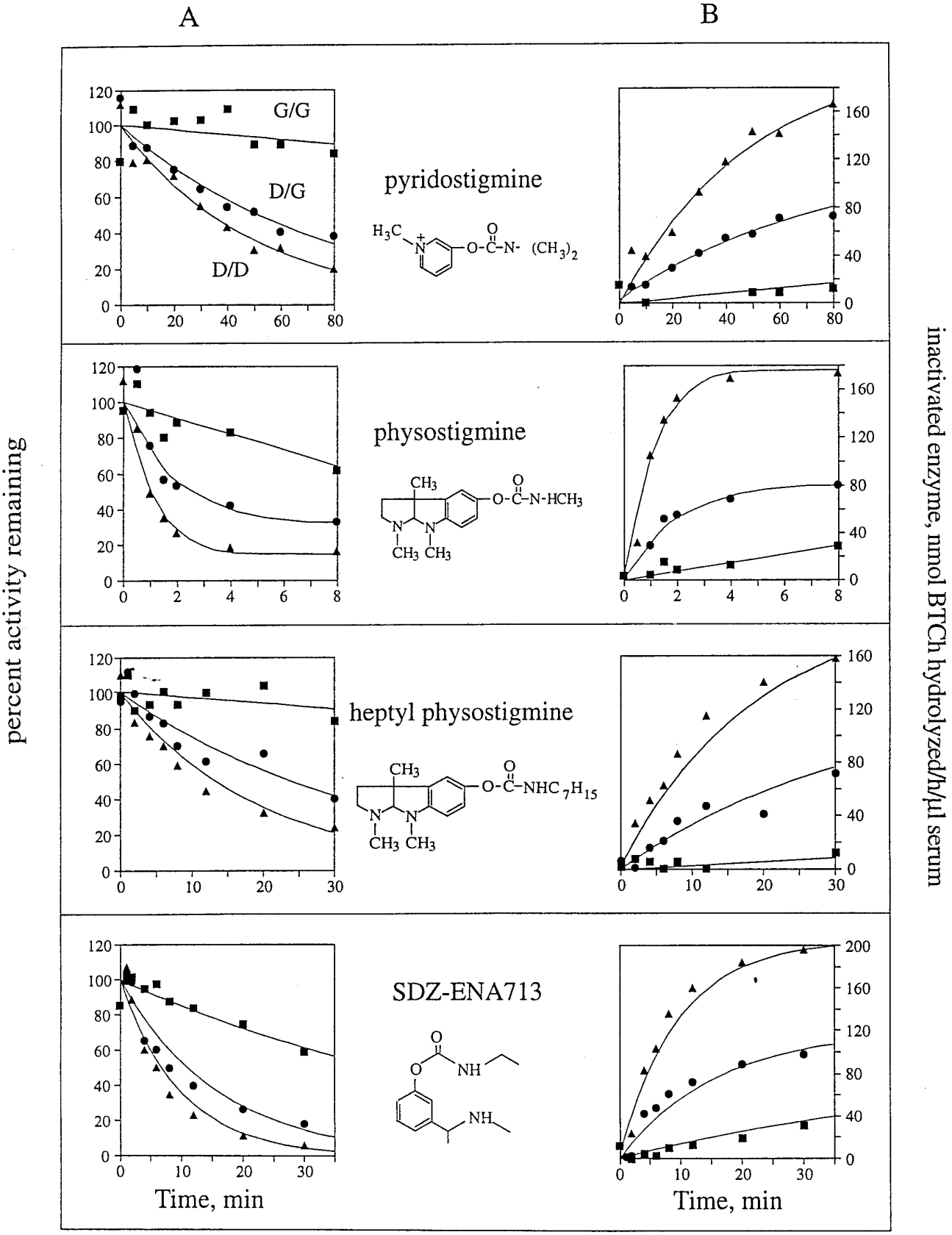

A
B
percent activity remaining
inactivated enzyme, nmol BTCh hydrolyzed/h/µl serum
G/G
D/G
D/D
pyridostigmine
physostigmine
heptyl physostigmine
SDZ-ENA713
Time, min
Time, min

Fig. 2:

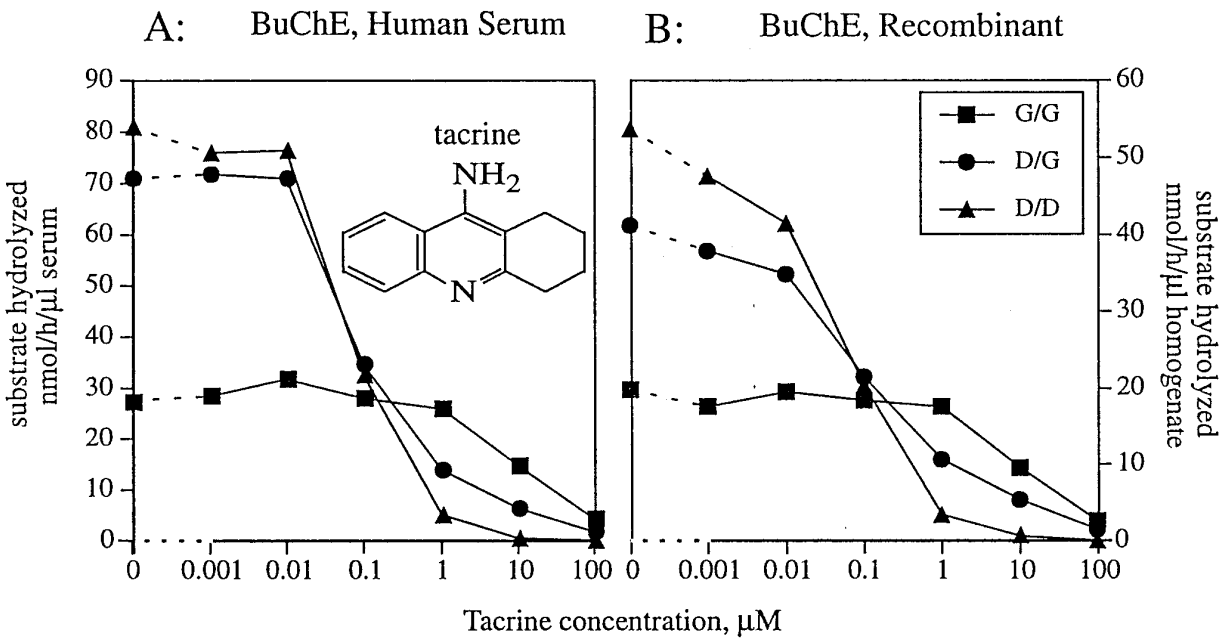

A:
BuChE, Human Serum
B:
BuChE, Recombinant
tacrine
NH2
N
G/G
D/G
D/D
substrate hydrolyzed
nmol/h/µl serum
substrate hydrolyzed
nmol/h/µl homogenate
0
10
20
30
40
50
60
70
80
90
0
0.001
0.01
0.1
1
10
100
Tacrine concentration, µM

Fig. 3:

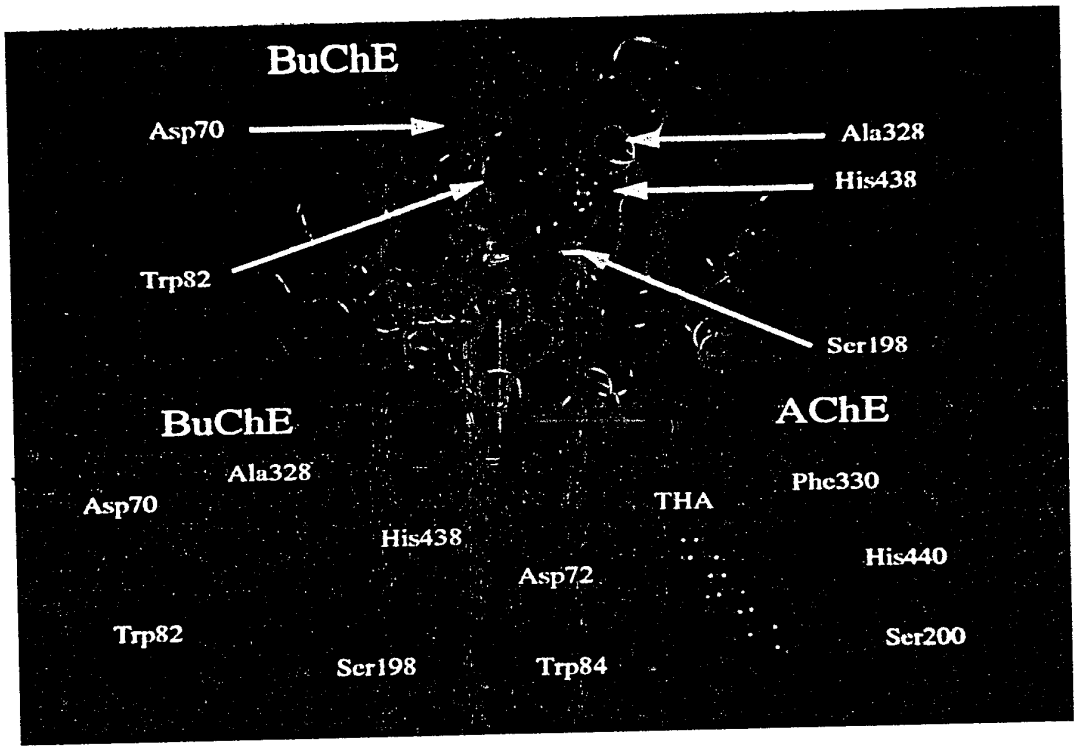
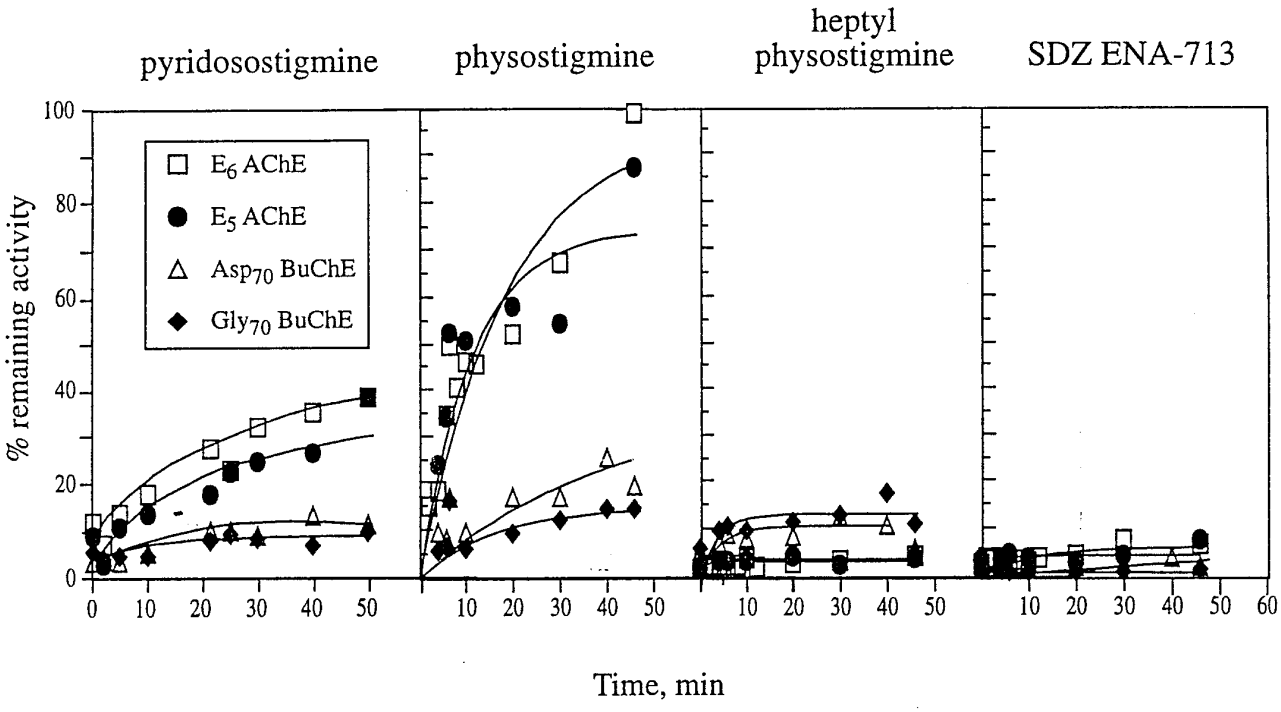

BuChE
Asp70
Ala328
His438
Trp82
Ser198
BuChE
AChE
Ala328
Asp70
Phe330
THA
His438
His440
Asp72
Trp82
Ser200
Ser198
Trp84

Fig. 4: Time dependent reactivation



Genetic predisposition to adverse consequences of anti-cholinesterase therapies in carriers of the "atypical" butyrylcholinesterase mutation

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Key Words

Acetylcholinesterase / Alzheimer's disease / Drug scavenging

Gulf War syndrome / Pyridostigmine / Tacrine

While butyrylcholinesterase (BuChE) attenuates the effects of anti-cholinesterases (anti-ChEs) by scavenging them[1], the influence of variant BuChEs[2] becomes urgent following the prophylactic use of the anti-ChE, pyridostigmine during the 1991 Gulf War in anticipation of nerve gas attack[3] and the approval of tacrine for treatment of Alzheimer's disease for improving residual cholinergic neurotransmission[4]. Adverse symptoms were reported for certain patients in both groups, but were difficult for interpretation[4,5]. Here, we report on an Israeli soldier, homozygous for "atypical" BuChE, who suffered severe symptoms under prophylactic pyridostigmine dosage during the Gulf War. His serum BuChE and recombinant "atypical" BuChE produced in microinjected oocytes[6] were far less sensitive than normal BuChE to inhibition by several carbamate anti-ChEs including pyridostigmine, and reacted with the reversible inhibitor tacrine with 200-fold lower affinity than normal BuChE or its related enzyme acetylcholinesterase (AChE). Variant BuChEs, with deficient scavenging, may thus cause adverse responses to anti-ChE therapies.

"Atypical" BCHE is the most common allele of the BCHE gene that causes a clinically variant phenotype. Due to substitution of aspartate at position 70 by glycine[6,7], the "atypical" enzyme is incapable of hydrolyzing succinylcholine administered at surgery[8,9], and is much less sensitive than the normal enzyme to several inhibitors[6-9]. Homozygous carriers of this variant allele (under 0.04% in Europe but up to 0.6% in certain middle-Eastern populations[10]) suffer post-anesthesia apnea[9] and hypersensitivity to the anti-ChE insecticide parathion[11]. Recently, we learned of an individual, A.B., who had experienced succinylcholine-induced apnea and, during the Gulf War under treatment with pyridostigmine, cholinergic symptoms. Therefore, we initiated a study of the interaction of ChE inhibitors in clinical use or testing[1] with serum ChEs from members of this subject's family, as compared to the enzyme from normal serum and with recombinantly produced variant ChEs[6].

BuChE from sera of A.B. and his father, previously identified as homozygous and heterozygous carriers of the "atypical" BCHE allele, respectively[10], were compared to BuChE

from individuals homozygous for the normal BCHE allele. To analyze inhibitors that covalently interact with ChEs, we immobilized native human BuChEs through monoclonal antibodies to multiwell microtiter plates[12]. Recombinant *Xenopus* oocyte-produced variant ChEs, including normal and "atypical" BuChEs[6] and brain- and blood cell- characteristic AChEs[13] were also subjected to inactivation by anti-ChEs and to subsequent spontaneous reactivation[12].

Normal BuChE hydrolyzed 81 ± 23 nmol butyrylthiocholine (BTCh)/hr/μl serum (assayed at 2 mM substrate, an average from 20 individuals). The activity of "atypical" BuChE was found to be about 3-fold lower in A.B.'s serum, prepared in 1994, which contained a normal amount of enzyme protein (approx. 20 ng/μl). This was seen also for recombinant "atypical" and normal BuChEs[6]. Heterozygotes presented intermediate specific activities, 60-70% of the values determined for normal homozygotes (average of 3 genotyped individuals). Inactivation measurements demonstrated that "atypical" BuChE reacts much slower than does its normal counterpart with four carbamates, pyridostigmine[3], physostigmine[14], heptyl physostigmine[15] and SDZ-ENA 713[16] (Table IA). Moreover, inactivation rate differences were also evident between the heterozygous enzyme and that of homozygous normal sera (Fig. 1A). The loss and rate of loss of BuChE activity, thus, depend significantly on the individual's genotype, and reveal drastically lower scavenging capacities for each of the tested drugs in sera of individuals with the "atypical" as compared to the normal alleles.

Dose-dependent inhibition by the reversible Alzhiemer's disease drug tacrine[4] was examined for native and recombinant *Xenopus* oocyte-produced normal and "atypical" monomeric BuChE (Table IB). To mimic the heterozygous state, we tested 1:1 mixtures of oocyte homogenates, expressing recombinant normal and "atypical" enzyme (approximately equal amounts of the ChEs) (Fig. 1B). In both cases, we observed a drastic reduction in the capacity of "atypical" BuChE, as compared with the normal enzyme, to interact with tacrine. The heterozygous conditions yielded the expected intermediary inhibition curves (Fig. 1B). The oocyte-produced enzyme remains primarily monomeric[11]. Therefore, the different scavenging capacities of BuChE variants depend exclusively upon the genotype of the

individual, as multisubunit assembly or competition between the two types of enzyme subunits, do not occur in oocytes and could not affect tacrine interactions. Analysis of the relevant site in the enzyme's three-dimensional structure[17], further revealed that the distance from the Asp70 carboxyl group to the tacrine anilinic nitrogen in BuChE is only 5.2 Å, indicating the possibility of a salt bridge (Fig. 2). This interaction is removed in "atypical" BuChE, reflected in a two orders of magnitude increase in tacrine's IC_{50}.

In addition to inactivation and removal rates and to general hemodynamic parameters, the dynamic level of drug concentrations in a patient's serum depends on the rate of reactivation of drug-enzyme complexes. The reactivation rates of AChE and BuChE differed for several drugs, with the decreasing order SDZ-ENA 713, heptyl physostigmine, pyridostigmine and physostigmine (Table IC). This is in agreement with the short therapeutic half-life of physostigmine[14] and the long half life of SDZ-ENA 713[16]. However, for no drug was there a dramatic difference between the blood and brain forms of AChE or between normal and "atypical" BuChE (Table IC), which indicates that the differential drug responses of "atypical" BuChE occur at the initial scavenging step.

The possibility that anti-ChE therapies may cause adverse reactions in individuals with variant BCHE genotypes, inferred by Valentino *et al.*, in their studies of charged anti-ChEs[18], becomes a pertinent issue in view of the administration of pyridostigmine bromide to over 400,000 Gulf War soldiers. This was probably the largest scale ever use of an investigational prophylactic drug, approved because of the anticipated exposure of those soldiers to nerve agents[3]. Clinical studies with pyridostigmine showed no adverse effects in volunteers, yet had been limited to small numbers of healthy males and to only several days exposure[5]. Also, war conditions included concurrent exposure to insecticides, chiefly organophosphorus anti-ChEs[19], which further increased the level of ChE inhibition in these soldiers to an unknown extent. In homozygotes and possibly in heterozygous carriers of the "atypical" BCHE allele, the lower capacity of blood BuChE to interact with and detoxify some of the drug should result in larger effective doses. "Atypical" homozygotes with practically none of the normal protective detoxifier, would hence become most vulnerable to AChE inhibition under treatment by any anti-ChE drug.

Several Alzheimer's disease drugs emerged in this study as much faster inactivators of BuChE than of AChE, suggesting that when administered to patients, these drugs will interact primarily with plasma BuChE. The clinically effective dose reaching the central nervous system depends heavily, therefore, on the BCHE genotype. Even without the complication of BCHE polymorphism, BuChE levels can vary with the general state of health[11]. Since Alzheimer's disease patients are far from being as healthy as the pyridostigmine-treated soldiers, they may present yet more drastic symptoms in response to inappropriate dosage of anti-ChEs. Significant differences are to be expected in homozygous "atypical"s and less so in heterozygotes, due to the over 10-fold differences in the inactivation rates by these drugs of normal and "atypical" BuChE. Tacrine, in particular, reacts with "atypical" BuChE so much more weakly than with its normal counterpart that the "atypical" enzyme becomes a negligible factor in attenuating its interaction with AChE. The reported high percentage of cholinergic symptoms under tacrine treatment (up to 15%[4]) may perhaps reflect BCHE heterozygotes and, in addition, patients with liver malfunction and consequently with low serum BuChE levels.

In addition to short-term effects, our findings may anticipate long-term adverse consequences of treatment with either type of anti-ChE in carriers of the "atypical" BCHE genotype. Such consequences may be associated with the Gulf War syndrome on one hand[20] or with tacrine's hepatotoxicity on the other[4]. These predictions can be tested by genotype/phenotype tests for "atypical" BCHE/BuChE[2,6,10]. If, indeed, the genotype of patients determines their response to anti-ChEs, as we predict based on the above experiments, the incidence of the "atypical" BCHE allele should be significantly higher among Alzheimer's disease patients with adverse short- and/or long-term reactions to tacrine and among veterans who experienced cholinergic effects and those who suffer from the Gulf War syndrome. Should this be the case, appropriate dosages of these drugs might be adjusted to match specific genotypes, for the purpose of avoiding these adverse responses.

Methodology

All procedures followed were in accord with the Helsinki Declaration as revised in 1983 and with local Institutional guidelines for the care and use of laboratory animals.

Patients and methods: A.B. (name and details in hospital Medical Records), born in 1970, suffered an incident of post-anesthesia apnea in 1989. Anesthesia was induced by Na pentothal (250 mg) and succinylcholine (75 mg) and benzodiazepam (5 mg), petidine (50 mg), beatryl (0.1 mg) and atropine (0.5 mg) were administered. Mechanical respiration was continued for 5 hr due to failure to breathe spontaneously after 30 min of surgery (closed reduction of bilateral tibial fractures). During this period the electrocardiogram and pulse remained normal, mean blood pressure was 130:80 and blood glucose, urea, Na and K values were normal. Hemoglobin count was 12 g/dl, with slight leukocytosis prior to surgery (14,000 cells/dl), yet with normal temperature. One day after surgery, the BuChE dibucaine number[9] was determined to be 23% of normal. A.B. was released following 9 days of uneventful hospitalization. We then found his serum BuChE to have approximately 30% of normal capacity for BTCh hydrolysis and no capacity for binding succinylcholine, suggesting that it was the "atypical" enzyme[6,7]. A.B. was diagnosed as being homozygous for the "atypical" allele, but not a carrier of other frequent point mutations of BuChE by PCR amplification, *SauIII*A restriction and direct sequencing of the corresponding region from the BCHE gene isolated from his venous blood DNA by established techniques[10]. The same methods revealed that both his parents and his sister were heterozygous carriers of the "atypical" BCHE allele. The patient was advised to avoid anti-ChE drugs or insecticides. A.B. served in the Israel Defense Forces in 1991, during the period of the Gulf War and, with others, thrice daily received 30 mg prophylactic doses of pyridostigmine. He developed nausea, insomnia, weight loss, and general fatigue, which worsened consistently, and a deep depression. Following discontinuation of pyridostigmine, his condition improved gradually over the following weeks. A.B. is currently without symptoms.

Variant enzymes: Serum BuChE activity against BTCh was measured spectrophotometrically as detailed previously[6]. Recombinant normal and "atypical" BuChEs were produced in *Xenopus* oocytes microinjected with *in vitro*-transcribed BCHEmRNAs prepared from the

corresponding cDNA types[6]. Alternative recombinant AChEs were produced in ACHEDNA-injected oocytes under control of the cytomegalovirus (CMV) promoter, using either the brain-characteristic 3'-exon 6[13] or the blood cell-expressed domain composed of the fourth pseudo-intron and the 3'-exon 5[13].

Inhibitors: Tacrine and physostigmine were purchased from Sigma Chemical Co. (St. Louis, MO). SDZ-ENA 713 and heptyl physostigmine were gifts of Sandoz (Bern, Switzerland) and Merck Sharp & Dohme (Harlow, U.K.), respectively. Pyridostigmine bromide was from Research Biochemicals International (Natik, MA).

Antibody immobilization's: Monoclonal mouse anti-human serum BuChE (no. 53-4) or anti-human AChE (no. 101-1) antibodies, 4 μg/ml, were adsorbed to multiwell microtiter plates overnight at 4 °C in carbonate buffer[12]. Free binding sites were blocked with PBS-T buffer (144 mM Na chloride, 20 mM Na phosphate, pH 7.4, 0.05% Tween 20, and 0.01% thimerosal) for 60 to 80 min at 37 °C. Homogenates of microinjected oocytes or serum samples were diluted 1:20 to 1:40 in PBS-T to achieve similar activity levels and were incubated in the antibody-coated wells for 4 hr at room temperature with agitation, and overnight at 4 °C. Plates were washed 3 times with PBS-T prior to use.

Inactivation and reactivation measurements: IC_{50} values were determined essentially as described[6], assaying soluble enzyme in the presence of tacrine against 1 mM acetylthiocholine. For inactivation by carbamates and reactivation, antibody-immobilized enzymes were exposed to the tested anti-ChEs in PBS-T buffer for varying times (0.5 to 80 min) at room temperature following an initial determination of catalytic activities. At the noted times, plates were washed 3 times with PBS-T and remaining substrate hydrolysis rates were determined[13]. Spontaneous reactivation at room temperature was measured for immobilized recombinant ChEs following complete inhibition, 3 washes with PBS-T, subsequent incubation and activity determination at 30 min.

Specific activity: These values were determined as detailed elsewhere[12].

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References

1. Schwarz M, Glick D, Loewenstein Y & Soreq H. Engineering of human cholinesterases explains and predicts diverse consequences of administration of various drugs and poisons. Pharmacol. Therap. (in press).
2. La Du BN, *et al.* Phenotypic and molecular biological analysis of human butyrylcholinesterase variants. Clin Biochem **23**,423-31 (1990).
3. Keeler JR, Hurst CG, Dunn MA. Pyridostigmine used as a nerve agent pretreatment under wartime conditions. J Am Med Assn **266**, 693-5 (1991).
4. Winker MA. Tacrine for Alzheimer's disease; which patient, what dose? J Am Med Assn **271**,1023-4 (1994).
5. Sharabi Y, *et al.* Survey of symptoms following intake of pyridostigmine during the Persian Gulf War. Isr J Med Sci **27**,656-8 (1991).
6. Neville LF. *et al.* Intramolecular relationships in cholinesterases revealed by oocyte expression of site-directed and natural variants of human BCHE. EMBO J **11**,1641-9 (1992).
7. McGuire MC, *et al.* Identification of the structural mutation responsible for the dibucaine-resistant (atypical) variant form of human serum cholinesterase. Proc Natl Acad Sci U S A **86**,953-7 (1989).
8. Kalow W & Davis RO. The activity of various esterase inhibitors towards atypical human serum cholinesterase. Biochem Pharmacol **1**,183-92 (1958).
9. Lockridge O. Genetic variants of serum cholinesterase influence metabolism of the muscle relaxant succinylcholine. Pharmacol Ther **47**,35-60 (1990).
10. Ehrlich G, *et al.* Population diversity and distinct haplotype frequencies associated with ACHE and BCHE genes of Israeli Jews from transcaucasian Georgia and from Europe. Genomics **22**,288-95 (1994).
11. Soreq H & Zakut H. Human Cholinesterases and Anticholinesterases. San Diego: Academic Press, 1993.

12. Schwarz, M. *et al.* Successive organophosphate inhibition and oxime reactivation reveals distinct responses of recombinant human cholinesterase variants. Molec Brain Res (in press).

13. Seidman, S. *et al.* Synaptic and epidermal accumulations of human acetylcholinesterase are encoded by alternative 3'-terminal exons. Mol Cell Biol (in press).

14. Giacobini E. The second generation of cholinesterase inhibitors: pharmacological aspects. In: Cholinergic Basis for Alzheimer Therapy, Becker R and Giacobini E, eds., Birkhauser, Boston, pp. 247-62 (1991).

15. Iversen LL. Approaches to cholinergic therapy in Alzheimer's disease. Prog Brain Res **98**,423-6 (1993).

16. Enz A, Amstutz R, Boddeke H, Gmelin G & Malonowski J. Brain selective inhibition of acetylcholinesterase: a novel approach to therapy for Alzheimer's disease. Prog Brain Res **98**,431-7 (1993).

17. Harel, M. *et al.* (1993) Quaternary ligand binding to aromatic residues in the active-site gorge of acetylcholinesterase. Proc. Natl. Acad. Sci. USA **90**,9031-5 (1993).

18. Valentino RJ, Lockridge O, Eckerson HW & La Du BN. Prediction of drug sensitivity in individuals with atypical serum cholinesterase based on *in vitro* biochemical studies. Biochem. Pharmacol. **30**,1643-9 (1981).

19. Gavageran, H. NIH panel rejects Persian Gulf Syndrom. Nature **369,** 8 (1994).

20. Harel M *et al.* Conversion of acetylcholinesterase to butyrylcholinesterase: modeling and mutagenesis. Proc Natl Acad Sci U S A. **89**,10827-31 (1992).

Legends For Figures

Fig. 1. Inhibition of BuChE variants by anti-ChEs.

A. Differential inactivation kinetics of variant Human sera BuChEs with carbamate inhibitors. Percent original activity as a function of time of exposure to 4 carbamates is presented for a representative experiment. Activities of immobilized ChEs were determined in 10 mM BTCh following incubation for the noted times with the covalently binding inhibitors pyridostigmine (10 μM), physostigmine (1 μM), heptyl physostigmine (0.01 μM), and SDZ-ENA 713 (10 μM). The lines are best fits of the data to first order conditions. ▲, normal, Asp70, BuChE homozygote (D/D); ■ "atypical", Gly70, homozygote (G/G); ●, BuChE heterozygote (G/D).

B. Inhibition of serum and recombinant BuChE by tacrine. In the left panel, data for 3 representative serum types inhibited by tacrine: serum of the propositus, A.B., a homozygote for the "atypical", Gly70, BuChE variant (G/G); of his father, a heterozygote for this variant (G/D); and of a normal homozygote with Asp70 (D/D). In the right panel, tacrine inhibition is observed on equivalent total amounts of recombinant normal (D/D) and "atypical" (G/G) BuChE, and a 1:1 mixture of the two (D/G).

Fig.2. ChE structure and tacrine binding . Upper: Three-dimensional ribbon structure of BuChE. The figure highlights several amino acid residues in space-filling models after Harel *et al.* (17, 20), using Insight II (Biosym Technologies, San Diego CA). The active site gorge lies in the plane of the figure, with its opening at the top. **Lower:** Residues surrounding tacrine (THA). The 3-dimensional structure of *Torpedo* AChE crystals soaked with tacrine (right), is compared to the homologous residues of BuChE (left).

Table I. Deficient interaction of "atypical" butyrylcholinesterase with various inhibitors

	BuChE		AChE	
	normal	"atypical"	brain-type (E6)	blood cell-type (E5)
A: second order inactivation rate constants ($M^{-1}min^{-1}$)[a]				
pyridostigmine (10^{-5} M)	1.9 ± 0.2	<0.8	22 ± 9	25 ± 7
physostigmine (10^{-6} M)	380 ± 160	26 ± 9	ND	ND
heptyl physostigmine (10^{-8} M)	11000 ± 3,000	770 ± 440	1600 ± 700	1400 ± 400
SDZ-ENA 713 (10^{-5} M)	14 ± 3	0.47 ± 0.46	4.3 ± 1.8	3.3 ± 0.6
B: IC_{50} values (μM)[b]				
tacrine (recombinant)	0.054 ± 0.036	11.4 ± 1.4	0.15 ± 0.08	0.15 ± 0.04
tacrine (serum)	0.082 ± 0.009	8.9 ± 2.5	------	------
C: Time dependent reactivation:[c]				
pyridostigmine	9	8	32	24
physostigmine	17	12	67	54
heptyl physostigmine	13	13	3	3
SDZ-ENA 713	2	1	8	4

Notes for Table I

[a]Pseudo first-order rate constants were extracted from data such as those in Fig. 1, but for the recombinant enzymes, and with the reagent concentrations indicated in parentheses, and were fit to a first order decay model using the least squares approach. Averages and standard deviations of at least 4 determinations are presented. ND, not determined because of the exceedingly fast reactivation rates.

[b]IC_{50} values of tacrine were measured for recombinant human ChEs and for sera in the presence of 1 mM BTCh for BuChE or 1 mM acetylthiocholine for AChE. Values are calculated by GraFit 3.0 (Erithacus Software, Staines, UK). ND, not determined. The data shown are averages and standard deviations of 2 serum samples or 3 recombinant enzyme samples.

[c]Spontaneous reactivation of recombinant human ChEs was examined after complete inhibition of the immobilised enzymes, followed by removal of unreacted inhibitor. Values of percent regained activity after 30 min are shown (average of two experiments).

Fig. 1A:

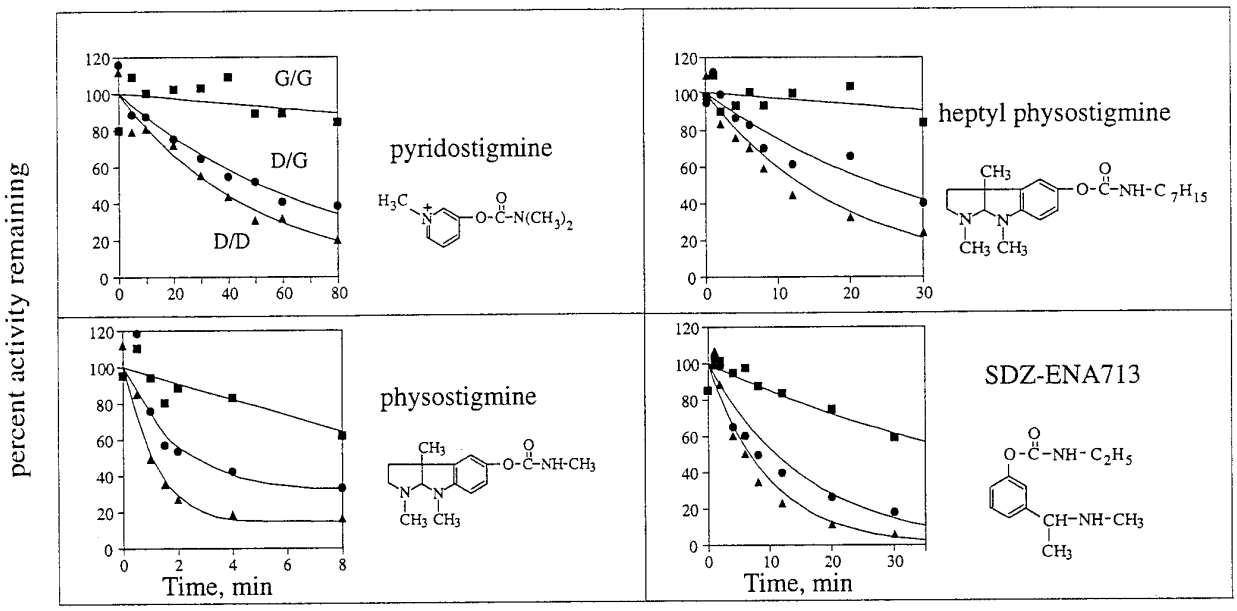

percent activity remaining
G/G
D/G
D/D
pyridostigmine
heptyl physostigmine
physostigmine
SDZ-ENA713
Time, min
Time, min

Fig. 1B:

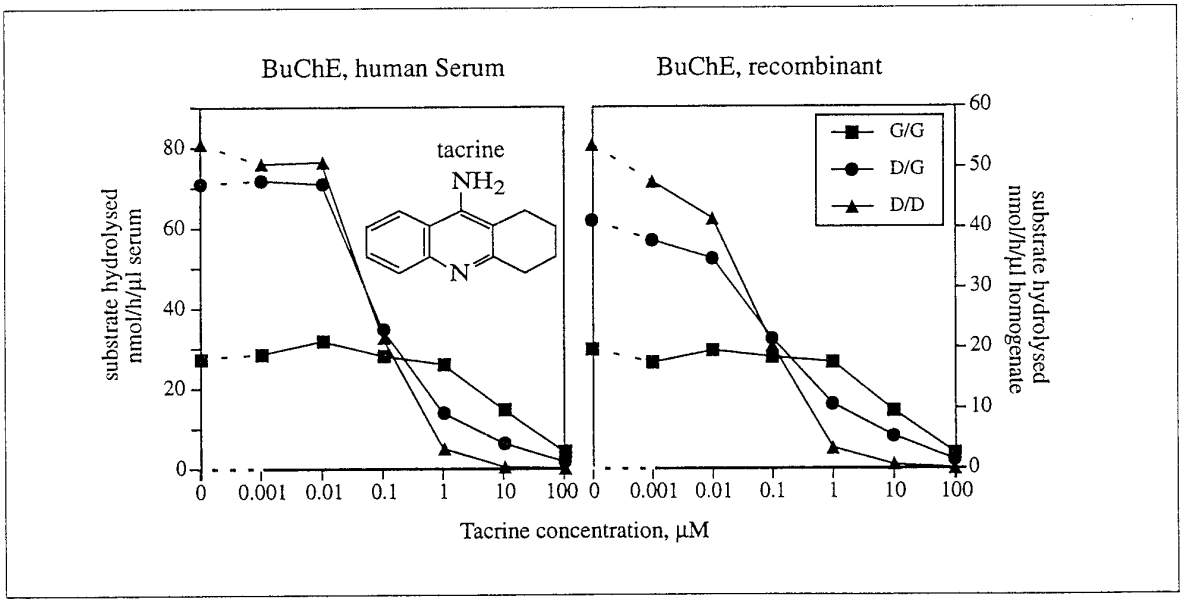
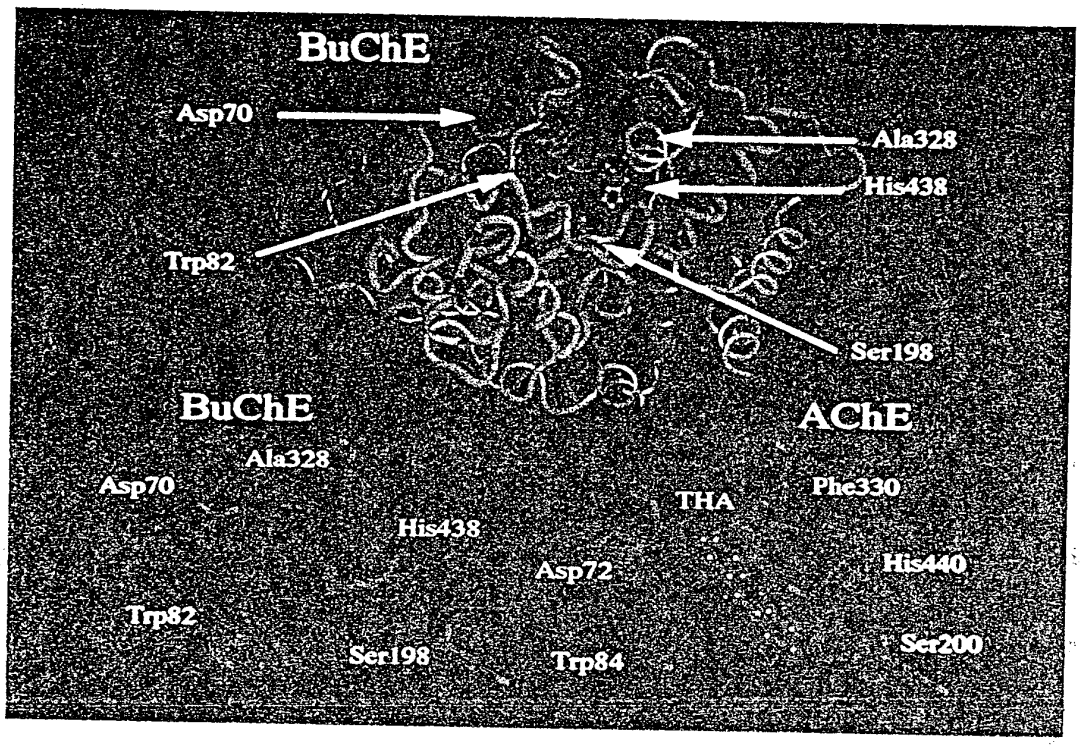

BuChE, human Serum
BuChE, recombinant
tacrine
G/G
D/G
D/D
substrate hydrolysed nmol/h/µl serum
substrate hydrolysed nmol/h/µl homogenate
Tacrine concentration, µM

Fig. 2:


BuChE
Asp70
Ala328
His438
Trp82
Ser198
BuChE
AChE
Ala328
Asp70
THA
Phe330
His438
Asp72
His440
Trp82
Ser200
Ser198
Trp84